PLANT *MSH2* SEQUENCES AND METHODS OF USE

FIELD OF THE INVENTION

The invention relates to the genetic manipulation of plants, particularly to modulating recombination and DNA repair mechanisms in plants.

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BACKGROUND OF THE INVENTION

Mismatched base pairing in DNA duplexes may arise due to errors introduced during DNA replication (Echols and Goodman (1991) *Annu. Rev. Biochem. 60*:477-511; Kornberg and Baker (1991) *DNA replication,* W.H. Freeman & Co., New York),

10   heteroduplex formation during homologous recombination (Holliday (1964) *Genet Res. 5*:282-304; Petes and Hill (1988) *Annu. Rev. Genet. 22*:147-168), as a consequence of mutation and by enzymatic modification of DNA such as deamination of 5-methylcytosine. Such mismatches can lead to genome instability. Therefore, all living systems have evolved specialized pathways to repair specific mismatches which are

15   somewhat different than other DNA repair mechanisms such as base excision repair and nucleotide excision repair (Wildenberg and Messelson (1975) *Proc. Natl. Acad. Sci. USA 72*:2202-2206; Wagner and Messelson (1996) *Proc. Natl. Acad. Sci. USA 73*:4136-4139; Radman and Wagner (1986) *Annu. Rev. Genet. 20*:523-538; Freidberg (1985) *DNA Repair*, W.H. Freeman & Co., New York). Early studies in prokaryotic systems,

20   especially *Escherichia coli*, led to the identification of one of these pathways, called the long-patch repair system or the methyl-directed mismatch-repair system (Radman and Wagner (1986) *Annu. Rev. Genet. 20*:523-538). This pathway exhibits rather broad specificities for repairing mismatches generated during DNA biosynthesis as well as recombination. Several genes essential for the methyl-directed mismatch repair have

25   been identified in *E. coli*. Primary among these are *mutS, mutL, mutH, UvrD*, and the

Dam methyltransferase and exonuclease genes (Freidberg (1985) *DNA Repair,* W.H. Freeman & Co., New York).

Genetic evidence for the existence of the mismatch-repair pathways in eukaryotes has been around since the late 1960s (Emerson (1969) *Genetic Organization* Caspari & Ravin, eds., Academic Press, New York, pp. 267-360). However, it was not until the early 1990s, following the first biochemical evidence for the repair activity in a eukaryote (Muster-Nassal and Kolodner (1986) *Proc. Natl. Acad. Sci. USA 83*:7618-7622) and the isolation and characterization of yeast *mutS* homologues, *Msh1* (Reenan and Kolodner (1992) *Genetics 132*:963-973), *MSH2* (Reenan and Kolodner (1992) *Genetics 132*:975-985) and *Msh3* (New *et al.* (1993) *Mol. Gen. Genet. 239*:97-108), that the existence of a mismatch-repair pathways in eukaryotes was clearly established. Subsequently, several eukaryotic *Msh* genes have been cloned and characterized (Nickoloff and Hoeskstra (1998) *DNA Damage and Repair*, vols. I-II, Humana Press, New York). Extensive and careful biochemical studies over the past decade have revealed that the gene products (denoted by MSH1, MSH2 etc.) of individual *Msh* gene family members exhibit remarkable specificity in their ability to participate in different biological processes. Thus, in yeast, MSH1 is primarily responsible for mitochondrial DNA repair, MSH2, MSH3, and MSH6 are involved in base mismatch repair and in modulating recombination, whereas MSH4 and MSH5 and are involved in modulating recombination. Precisely how MSH2, MSH3, and MSH6 participate in recombination has not yet been determined. It has been proposed that in addition to their mismatch-repair activity, these gene products interact with other cellular components involved in resolution of the Holliday junction (Nickoloff and Hoeskstra (1998) *DNA Damage and Repair*, vols. I-II, Humana Press, New York).

Interestingly, in a recent study with mammalian cells, mismatch repair has been shown to have an anti-recombinational effect. Thus, in a mouse *msh2* cell line, target integration of a plasmid DNA at the Rb locus was increased 50-fold. Furthermore, *MSH2* and *Msh3* homologues are known to be involved in gene targeting and gene modification processes (deWind *et al.* (1995) *Cell 82*:321-330). The deWind reference, as well as the Abuin *et al.* ((2000) *Cellular Biol. 20*: 149-157), disclose that MSH2 deficiency increases

the recombination frequency between non-identical DNA substrates. The anti-recombination effect is only observed with non-identical DNAs; recombination between identical DNA substrates is unaffected in msh2 lines. This is likely because pairing of homologous DNA sequences does not lead to DNA mismatches.

5   A combination of factors appears to render plant genomes highly susceptible to mutation. Complex genomes of higher plants contain large numbers of putative mutational hotspots, such as microsatellites, repeated elements and 5-methylcytosine. In addition, unlike many other multicellular organisms, plant germ cells are derived from somatic progenitors that have undergone many cell divisions. High DNA replication

10   fidelity is crucial to the faithful transmission of genetic information to subsequent plant generations. The DNA mismatch-repair system plays a crucial role in maintaining the integrity of the genome. Mismatch-repair activities identify and catalyze the repair of DNA polymerase errors and base-pair mismatches and act to restrict recombination between non-homologous DNA sequences. The proofreading and anti-recombination

15   functions of mismatch-repair activities likely play a key role in the fitness of subsequent plant generations.

        The methyl-directed mismatch-repair system of *E. coli* is well characterized. For review, see Modrich and Lahue ((1996) *Annu. Rev. Biochem. 65*:101-133). In brief, the key components in *E. coli* mismatch repair are: MutS, which interacts directly with

20   mismatched DNA; and MutL, which, through its interaction with MutS, activates the MutH endonuclease. Upon activation, MutH endonuclease introduces a nick in the unmethylated DNA flanking the site of the base-pair mismatch at a hemi-methylated GATC site. The nicked strand is then degraded through the site of the mismatch and the degraded sequences are resynthesized and ligated.

25   Eukaryotes encode a family of MutS orthologs or homologs, known as *Msh*. Mismatch recognition in eukaryotes is accomplished by a heterodimer of MSH proteins, depending upon the type of mismatch. Heterodimers of MSH2 and MSH3 recognize insertion mismtaches and DNA loops, while heterodimers of MSH2 and MSH6 interact preferentially with base-pair mismatches and single base insertions. (Marsischky *et al.*

30   (1996) *Genes Devel. 10*:407-420) MSH2 is the key component in mismatch recognition,

because it is required to initiate correction of any sort of mismatch. Biochemical and genetic studies in *E. coli* have demonstrated an antirecombination activity associated with homologs of MutS (Rayssiguier *et al.* (1989) *Nature 342*:396-401). The role of MSH2 in preventing recombination between partially homologous (homeologous) sequences has

5 also been established in *S. cerevisiae* (Alani *et al.* (1994) *Genetics. 137*:19-39).

While much is known about the biochemical nature of DNA mismatch repair in bacterial, yeast, and mammalian systems, very little is known about the corresponding repair pathways in plants. MutS homolog genes have been identified in a number of plant species, including *Arabidopsis*, maize and wheat, but the contributions of these

10 proteins to genome stability and DNA proofreading has not been established.

SUMMARY OF THE INVENTION

Compositions and methods for altering mismatch repair and recombination frequency are provided. Such compositions and methods find use in altering mutation

15 rates, recombination frequencies and DNA repair processes, in producing dominant-negative *MSH2* polynucleotides and the polypeptides encoded thereby, and in improving the efficiency of transformation and chimeraplasty, in both eukaryotic and prokaryotic organisms. The compositions comprise isolated nucleic acid molecules comprising nucleotide sequences encoding tobacco MSH2 proteins, nucleotide sequences that encode

20 dominant-negative MSH2 variants, and the proteins encoded by such nucleotide sequences. Further provided are expression cassettes comprising an *MSH2* nucleotide sequence of the invention operably linked to a promoter that drives expression in an organism of interest. The methods involve introducing into an organism an *MSH2* nucleotide sequence of the invention operably linked to a promoter that drives expression

25 in the organism or alternatively introducing an MSH2 protein into the organism. If decreased expression is desired, the methods can additionally involve co-suppression, antisense suppression, or a dominant-negative approach.

Additionally provided are nucleic acid molecules sequences comprising nucleotide sequences of promoters of tobacco *MSH2* genes. Expression cassettes

30 comprising such *MSH2* promoter sequences are also provided. The promoter nucleotide

sequences of the invention find use in methods for regulating the expression of a heterologous nucleotide sequence of interest in a plant. The methods involve introducing in the genome of a plant a nucleotide construct comprising an *MSH2* promoter nucleotide sequence operably linked to a heterologous sequence.

5   Transformed host cells, transformed plants, tissues, cells and seeds thereof are provided.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 summarizes the nucleotide polymorphisms identified in *NtMSH2* (*Nicotiana tabacum MSH2*) cDNA and genomic sequences. Degenerate polymorphisms

10   are present at nucleotide positions 36, 48, 81, 93 and 144 with respect to the translational start. The nucleotide polymorphism at position 145 is non-degenerate. *NtMSH2A1*, *NtMSH2A2* and *NtMSH2A3* encode serine, while *NtMSH2B1* encodes alanine. Single letter amino acid abbreviations are used.

DETAILED DESCRIPTION OF THE INVENTION

15   The invention is drawn to the processes that living organisms use to maintain the fidelity of DNA during replication and recombination. In particular, the invention provides isolated nucleic acid molecules comprising nucleotide sequences which encode MutS homologues (MSH) from tobacco, particularly MSH2, and the isolated proteins

20   encoded by such nucleotide sequences. Such nucleotide sequences find use in plants and other organisms in altering the frequency of recombination and the efficiency of gene modification processes such as, for example, chimeraplasty. The invention further provides isolated nucleotide molecules comprising promoters of the tobacco *MSH2* genes. Such promoters find use in regulating gene expression in plants.

25   Compositions of the invention include nucleotide sequences from genes that encode proteins known as MSH2 proteins. Such proteins are known to be involved in the processes of DNA repair and recombination. In particular, the present invention provides for isolated nucleic acid molecules comprising nucleotide sequences encoding the amino acid sequences shown in SEQ ID NOs: 2 and 4, or the nucleotide sequences encoding the

30   DNA sequences deposited in a bacterial host as Patent Deposit Nos. PTA-1889 and PTA-

1890. Further provided are polypeptides having an amino acid sequence encoded by a nucleic acid molecule described herein, for example those set forth in SEQ ID NOs: 1 and 3 those deposited in a bacterial host as Patent Deposit Nos. PTA-1889 and PTA-1890, and fragments and variants thereof.

5   Plasmids containing the nucleotide sequences (SEQ ID NOS:1 and 3) of the invention were deposited with the Patent Depository of the American Type Culture Collection (ATCC), 10801 University Blvd., Manassas, Virginia, on May 18, 2000 and assigned Patent Deposit Nos. PTA-1889 and PTA-1890. These deposits will be maintained under the terms of the Budapest Treaty on the International Recognition of

10   the Deposit of Microorganisms for the Purposes of Patent Procedure. These deposits were made merely as a convenience for those of skill in the art and are not an admission that a deposit is required under 35 U.S.C. § 112.

         The invention encompasses isolated or substantially purified nucleic acid or protein compositions. An "isolated" or "purified" nucleic acid molecule or protein, or

15   biologically active portion thereof, is substantially free of other cellular material, or culture medium when produced by recombinant techniques, or substantially free of chemical precursors or other chemicals when chemically synthesized. Preferably, an "isolated" nucleic acid is free of sequences (preferably protein encoding sequences) that naturally flank the nucleic acid (i.e., sequences located at the 5' and 3' ends of the nucleic

20   acid) in the genomic DNA of the organism from which the nucleic acid is derived. For example, in various embodiments, the isolated nucleic acid molecule can contain less than about 5 kb, 4 kb, 3 kb, 2 kb, 1 kb, 0.5 kb, or 0.1 kb of nucleotide sequences that naturally flank the nucleic acid molecule in genomic DNA of the cell from which the nucleic acid is derived. A protein that is substantially free of cellular material includes

25   preparations of protein having less than about 30%, 20%, 10%, 5%, (by dry weight) of contaminating protein. When the protein of the invention or biologically active portion thereof is recombinantly produced, preferably culture medium represents less than about 30%, 20%, 10%, or 5% (by dry weight) of chemical precursors or non-protein-of-interest chemicals.

Fragments and variants of the disclosed nucleotide sequences and proteins encoded thereby are also encompassed by the present invention. By "fragment" is intended a portion of the nucleotide sequence or a portion of the amino acid sequence and hence protein encoded thereby. Fragments of a nucleotide sequence may encode protein

5   fragments that retain the biological activity of the native protein and hence mismatch-repair activity. Alternatively, fragments of a nucleotide sequence that are useful as hybridization probes generally do not encode fragment proteins retaining biological activity. Thus, fragments of a nucleotide sequence may range from at least about 20 nucleotides, about 50 nucleotides, about 100 nucleotides, and up to the full-length

10   nucleotide sequence encoding the proteins of the invention.

A fragment of an *MSH2* nucleotide sequence that encodes a biologically active portion of an MSH2 protein of the invention will encode at least 15, 25, 30, 50, 100, 150, 200, 250, 300, 350, 400, 450, 500, 550, 600, 650, 700, 750, 800, 850, or 900 contiguous amino acids, or up to the total number of amino acids present in a full-length MSH2

15   protein of the invention (for example, 939 amino acids for each of SEQ ID NOS:2 and 4, respectively). Fragments of an *MSH2* nucleotide sequence that are useful as hybridization probes or PCR primers generally need not encode a biologically active portion of an MSH2 protein.

Thus, a fragment of an *MSH2* nucleotide sequence may encode a biologically

20   active portion of an MSH2 protein, or it may be a fragment that can be used as a hybridization probe or PCR primer using methods disclosed below. A biologically active portion of an MSH2 protein can be prepared by isolating a portion of one of the *MSH2* nucleotide sequences of the invention, expressing the encoded portion of the MSH2 protein (e.g., by recombinant expression *in vitro*), and assessing the activity of the

25   encoded portion of the MSH2 protein. Nucleic acid molecules that are fragments of an *Msh* nucleotide sequence comprise at least 16, 20, 50, 75, 100, 150, 200, 250, 300, 350, 400, 450, 500, 550, 600, 650, 700, 800, 900, 1,000, 1,100, 1,200, 1,300, 1,400, 1,500, 1,750, 2,000, 2,250, 2,500, 2,750, or 3,000 nucleotides, or up to the number of nucleotides present in a full-length *MSH2* nucleotide sequence disclosed herein (for

30   example, 3033 nucleotides for each of SEQ ID NOS: 1 and 3).

Similarly, a fragment of an *MSH2* nucleotide sequence may encode a biologically active portion of a promoter that is capable of driving the expression of an operably linked nucleotide sequence in a plant or it may be a fragment that can be used as a hybridization probe or PCR primer using methods disclosed below. A biologically active

5   portion of an *Msh2* promoter can be prepared by isolating a portion of one of the *MSH2* promoter sequences of the invention, operably linking the promoter sequence to a nucleotide sequence such as, for example, a reporter gene, transforming a plant cell with the operably linked construct, and assessing the activity of the *MSH2* promoter in the plant cell. Nucleic acid molecules that are fragments of an *MSH2* nucleotide sequence

10   comprise at least 16, 20, 50, 75, 100, 150, or 160 nucleotides, or up to the number of nucleotides present in a full-length *MSH2* promoter sequence disclosed herein (for example, 160, 163, 163, 165, 166, 166, 166, 165, nucleotides for SEQ ID NOS: 5, 6, 7, 8, 9, 10, 11, and 12, respectively).

By "variants" is intended substantially similar sequences. For nucleotide

15   sequences, conservative variants include those sequences that, because of the degeneracy of the genetic code, encode the amino acid sequence of one of the MSH2 polypeptides of the invention. Naturally occurring allelic variants such as these can be identified with the use of well-known molecular biology techniques, as, for example, with polymerase chain reaction (PCR) and hybridization techniques as outlined below. Variant nucleotide

20   sequences also include synthetically derived nucleotide sequences, such as those generated, for example, by using site-directed mutagenesis but which still encode an MSH2 protein of the invention, or in the case of promoter sequences, retain the capability of driving the expression of an operably linked nucleotide sequence in a plant. Generally, variants of a particular nucleotide sequence of the invention will have at least about 70%,

25   generally at least about 75%, 80%, 85%, preferably at least about 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, and more preferably at least about 98%, 99% or more sequence identity to that particular nucleotide sequence as determined by sequence alignment programs described elsewhere herein using default parameters.

By "variant" protein is intended a protein derived from the native protein by

30   deletion (so-called truncation) or addition of one or more amino acids to the N-terminal

and/or C-terminal end of the native protein; deletion or addition of one or more amino acids at one or more sites in the native protein; or substitution of one or more amino acids at one or more sites in the native protein. Variant proteins encompassed by the present invention are biologically active, that is they continue to possess the desired biological

5   activity of the native protein, that is, mismatch-repair activity as described herein. Such variants may result from, for example, genetic polymorphism or from human manipulation. Biologically active variants of a native MSH2 protein of the invention will have at least about 70%, generally at least about 75%, 80%, 85%, preferably at least about 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, and more preferably at least about

10 98%, 99% or more sequence identity to the amino acid sequence for the native protein as determined by sequence alignment programs described elsewhere herein using default parameters. A biologically active variant of a protein of the invention may differ from that protein by as few as 1-15 amino acid residues, as few as 1-10, such as 6-10, as few as 5, as few as 4, 3, 2, or even 1 amino acid residue.

15   The proteins of the invention may be altered in various ways including amino acid substitutions, deletions, truncations, and insertions. Methods for such manipulations are generally known in the art. For example, amino acid sequence variants of the MSH2 proteins can be prepared by mutations in the DNA. Methods for mutagenesis and nucleotide sequence alterations are well known in the art. See, for example, Kunkel

20 (1985) *Proc. Natl. Acad. Sci. USA 82*:488-492; Kunkel *et al.* (1987) *Methods in Enzymol. 154*:367-382; US Patent No. 4,873,192; Walker and Gaastra, eds. (1983) *Techniques in Molecular Biology* (MacMillan Publishing Company, New York) and the references cited therein. Guidance as to appropriate amino acid substitutions that do not affect biological activity of the protein of interest may be found in the model of Dayhoff *et al.* (1978)

25 *Atlas of Protein Sequence and Structure* (Natl. Biomed. Res. Found., Washington, D.C.), herein incorporated by reference. Conservative substitutions, such as exchanging one amino acid with another having similar properties, may be preferable.

        Thus, the genes and nucleotide sequences of the invention include both the naturally occurring sequences as well as mutant forms. Likewise, the proteins of the

30 invention encompass both naturally occurring proteins as well as variations and modified

forms thereof. Such variants will continue to possess the desired MSH2 activity, the mutations that will be made in the DNA encoding the variant must not place the sequence out of reading frame and preferably will not create complementary regions that could produce secondary mRNA structure. See, EP Patent Application Publication No. 75,444.

5   Additionally, the proteins of the invention also encompass fragments and variants that can be in dominant-negative strategies for reducing the biological activity of a MutS homologue or MSH2. Such dominant-negative fragments and variants of the MSH2 proteins of the invention, when expressed in a cell, are capable of reducing the biological activity of a MutS homologue or MSH2 therein. It is recognized that such dominant-

10   negative variants can be full-length MSH2 proteins or can be truncated forms. The invention also encompasses the nucleotide sequences which encode these dominant-negative fragments and variants

       The deletions, insertions, and substitutions of the protein sequences encompassed herein are not expected to produce radical changes in the characteristics of the protein.

15   However, when it is difficult to predict the exact effect of the substitution, deletion, or insertion in advance of doing so, one skilled in the art will appreciate that the effect will be evaluated by routine screening assays. That is, the activity can be evaluated by one or methods known in the art including, but not limited to, gel shift assays to demonstrate binding to specific mismatched substrates, in vitro mismatch repair assays, and in vivo

20   mismatch repair assays, such as, for example, determination of in vivo microsatellite stability and monitoring spontaneous mutation rates. See, for example, Marsischky *et al.* (1996) *Genes Devel. 10*:407-420; deWind *et al.* (1995) *Cell 82*:321-330; Holmes *et al.* (1990) *Proc. Natl. Acad. Sci USA 87*: 5837-5841; Reenan and Kolodner (1992) *Genetics 132*:963-973 See, Su *et al.* (1988*) J. Biol. Chem.* 263:6829-6835; Holmes *et al.* (1990)

25   *Proc. Natl. Acad. Sci.* USA 87:5837-5841; and Rice *et al.* (2000) *Plant Physiol.* 123:427-438; all of which are hereby herein incorporated by reference.

       Variant nucleotide sequences and proteins also encompass sequences and proteins derived from a mutagenic and/or recombinogenic procedure such as DNA shuffling. With such a procedure, one or more different MSH2 coding sequences can be

30   manipulated to create a new MSH2 possessing the desired properties. In this manner,

libraries of recombinant polynucleotides are generated from a population of related sequence polynucleotides comprising sequence regions that have substantial sequence identity and can be homologously recombined *in vitro* or *in vivo*. For example, using this approach, sequence motifs encoding a domain of interest may be shuffled between the

5   *Msh* gene of the invention and other known *Msh* genes to obtain a new gene coding for a protein with an improved property of interest, such as an increased K_m in the case of an enzyme. Strategies for such DNA shuffling are known in the art. See, for example, Stemmer (1994) *Proc. Natl. Acad. Sci. USA 91*:10747-10751; Stemmer (1994) *Nature 370*:389-391; Crameri *et al.* (1997) *Nature Biotech. 15*:436-438; Moore *et al.* (1997) *J.*

10   *Mol. Biol. 272*:336-347; Zhang *et al. (1997) Proc. Natl. Acad. Sci. USA 94*:4504-4509; Crameri *et al.* (1998) *Nature 391*:288-291; and U.S. Patent Nos. 5,605,793 and 5,837,458.

The nucleotide sequences of the invention can be used to isolate corresponding sequences from other organisms, particularly other plants. In this manner, methods such

15   as PCR, hybridization, and the like can be used to identify such sequences based on their sequence homology to the sequences set forth herein. Sequences isolated based on their sequence identity to the entire *MSH2* sequences set forth herein or to fragments thereof are encompassed by the present invention. Such sequences include sequences that are orthologs of the disclosed sequences. By "orthologs" is intended genes derived from a

20   common ancestral gene and which are found in different species as a result of speciation. Genes found in different species are considered orthologs when their nucleotide sequences and/or their encoded protein sequences share substantial identity as defined elsewhere herein. Functions of orthologs are often highly conserved among species.

In a PCR approach, oligonucleotide primers can be designed for use in PCR

25   reactions to amplify corresponding DNA sequences from cDNA or genomic DNA extracted from any plant of interest. Methods for designing PCR primers and PCR cloning are generally known in the art and are disclosed in Sambrook *et al.* (1989) *Molecular Cloning: A Laboratory Manual* (2d ed., Cold Spring Harbor Laboratory Press, Plainview, New York). See also Innis *et al.*, eds. (1990) *PCR Protocols: A Guide to*

30   *Methods and Applications* (Academic Press, New York); Innis and Gelfand, eds. (1995)

PCR Strategies (Academic Press, New York); and Innis and Gelfand, eds. (1999) *PCR Methods Manual* (Academic Press, New York). Known methods of PCR include, but are not limited to, methods using paired primers, nested primers, single specific primers, degenerate primers, gene-specific primers, vector-specific primers, partially-mismatched

5   primers, and the like.

In hybridization techniques, all or part of a known nucleotide sequence is used as a probe that selectively hybridizes to other corresponding nucleotide sequences present in a population of cloned genomic DNA fragments or cDNA fragments (i.e., genomic or cDNA libraries) from a chosen organism. The hybridization probes may be genomic

10   DNA fragments, cDNA fragments, RNA fragments, or other oligonucleotides, and may be labeled with a detectable group such as ^{32}P, or any other detectable marker. Thus, for example, probes for hybridization can be made by labeling synthetic oligonucleotides based on the *MSH2* sequences of the invention. Methods for preparation of probes for hybridization and for construction of cDNA and genomic libraries are generally known in

15   the art and are disclosed in Sambrook *et al.* (1989) *Molecular Cloning: A Laboratory Manual* (2d ed., Cold Spring Harbor Laboratory Press, Plainview, New York).

For example, an entire *MSH2* sequence disclosed herein, or one or more portions thereof, may be used as a probe capable of specifically hybridizing to corresponding *MSH2* sequences and messenger RNAs. To achieve specific hybridization under a

20   variety of conditions, such probes include sequences that are unique among *MSH2* sequences and are preferably at least about 10 nucleotides in length, and most preferably at least about 20 nucleotides in length. Such probes may be used to amplify corresponding *MSH2* sequences from a chosen plant by PCR. This technique may be used to isolate additional coding sequences from a desired plant or as a diagnostic assay

25   to determine the presence of coding sequences in a plant. Hybridization techniques include hybridization screening of plated DNA libraries (either plaques or colonies; see, for example, Sambrook *et al.* (1989) *Molecular Cloning: A Laboratory Manual* (2d ed., Cold Spring Harbor Laboratory Press, Plainview, New York).

Hybridization of such sequences may be carried out under stringent conditions.

30   By "stringent conditions" or "stringent hybridization conditions" is intended conditions

under which a probe will hybridize to its target sequence to a detectably greater degree than to other sequences (e.g., at least 2-fold over background). Stringent conditions are sequence-dependent and will be different in different circumstances. By controlling the stringency of the hybridization and/or washing conditions, target sequences that are 100%

5   complementary to the probe can be identified (homologous probing). Alternatively, stringency conditions can be adjusted to allow some mismatching in sequences so that lower degrees of similarity are detected (heterologous probing). Generally, a probe is less than about 1000 nucleotides in length, preferably less than 500 nucleotides in length.

Typically, stringent conditions will be those in which the salt concentration is less

10   than about 1.5 M Na ion, typically about 0.01 to 1.0 M Na ion concentration (or other salts) at pH 7.0 to 8.3 and the temperature is at least about 30°C for short probes (e.g., 10 to 50 nucleotides) and at least about 60°C for long probes (e.g., greater than 50 nucleotides). Stringent conditions may also be achieved with the addition of destabilizing agents such as formamide. Exemplary low stringency conditions include

15   hybridization with a buffer solution of 30 to 35% formamide, 1 M NaCl, 1% SDS (sodium dodecyl sulphate) at 37°C, and a wash in 1X to 2X SSC (20X SSC = 3.0 M NaCl/0.3 M trisodium citrate) at 50 to 55°C. Exemplary moderate stringency conditions include hybridization in 40 to 45% formamide, 1.0 M NaCl, 1% SDS at 37°C, and a wash in 0.5X to 1X SSC at 55 to 60°C. Exemplary high stringency conditions include

20   hybridization in 50% formamide, 1 M NaCl, 1% SDS at 37°C, and a wash in 0.1X SSC at 60 to 65°C. The duration of hybridization is generally less than about 24 hours, usually about 4 to about 12 hours.

Specificity is typically the function of post-hybridization washes, the critical factors being the ionic strength and temperature of the final wash solution. For DNA-

25   DNA hybrids, the T_m can be approximated from the equation of Meinkoth and Wahl (1984) *Anal. Biochem. 138*:267-284: $T_m = 81.5°C + 16.6 (\log M) + 0.41 (\%GC) - 0.61$ (% form) $- 500/L$; where M is the molarity of monovalent cations, %GC is the percentage of guanosine and cytosine nucleotides in the DNA, % form is the percentage of formamide in the hybridization solution, and L is the length of the hybrid in base pairs.

30   The T_m is the temperature (under defined ionic strength and pH) at which 50% of a

complementary target sequence hybridizes to a perfectly matched probe. T_m is reduced by about 1°C for each 1% of mismatching; thus, T_m, hybridization, and/or wash conditions can be adjusted to hybridize to sequences of the desired identity. For example, if sequences with $\geq$90% identity are sought, the T_m can be decreased 10°C. Generally,

5   stringent conditions are selected to be about 5°C lower than the thermal melting point (T_m) for the specific sequence and its complement at a defined ionic strength and pH. However, severely stringent conditions can utilize a hybridization and/or wash at 1, 2, 3, or 4°C lower than the thermal melting point (T_m); moderately stringent conditions can utilize a hybridization and/or wash at 6, 7, 8, 9, or 10°C lower than the thermal melting

10   point (T_m); low stringency conditions can utilize a hybridization and/or wash at 11, 12, 13, 14, 15, or 20°C lower than the thermal melting point (T_m). Using the equation, hybridization and wash compositions, and desired T_m, those of ordinary skill will understand that variations in the stringency of hybridization and/or wash solutions are inherently described. If the desired degree of mismatching results in a T_m of less than

15   45°C (aqueous solution) or 32°C (formamide solution), it is preferred to increase the SSC concentration so that a higher temperature can be used. An extensive guide to the hybridization of nucleic acids is found in Tijssen (1993) *Laboratory Techniques in Biochemistry and Molecular Biology—Hybridization with Nucleic Acid Probes,* Part I, Chapter 2 (Elsevier, New York); and Ausubel *et al.*, eds. (1995) *Current Protocols in*

20   *Molecular Biology*, Chapter 2 (Greene Publishing and Wiley-Interscience, New York). See Sambrook *et al.* (1989) *Molecular Cloning: A Laboratory Manual* (2d ed., Cold Spring Harbor Laboratory Press, Plainview, New York).

Thus, isolated sequences that encode of an MSH2 protein and which hybridize under stringent conditions to the *MSH2* sequences disclosed herein, or to fragments

25   thereof, are encompassed by the present invention.

The following terms are used to describe the sequence relationships between two or more nucleic acids or polynucleotides: (a) "reference sequence", (b) "comparison window", (c) "sequence identity", (d) "percentage of sequence identity", and (e) "substantial identity".

(a)   As used herein, "reference sequence" is a defined sequence used as a basis for sequence comparison. A reference sequence may be a subset or the entirety of a specified sequence; for example, as a segment of a full-length cDNA or gene sequence, or the complete cDNA or gene sequence.

5   (b)   As used herein, "comparison window" makes reference to a contiguous and specified segment of a polynucleotide or polypeptide sequence, wherein the polynucleotide or polypeptide sequence in the comparison window may comprise additions or deletions (i.e., gaps) compared to the reference sequence (which does not comprise additions or deletions) for optimal alignment of the two sequences. Generally,

10   the comparison window is at least 20 contiguous nucleotides or amino acids in length, and optionally can be 30, 40, 50, 100, or longer. Those of skill in the art understand that to avoid a high similarity to a reference sequence due to inclusion of gaps in the polynucleotide or polypeptide sequence a gap penalty is typically introduced and is subtracted from the number of matches.

15   Methods of alignment of sequences for comparison are well known in the art. Thus, the determination of percent sequence identity between any two sequences can be accomplished using a mathematical algorithm. Preferred, non-limiting examples of such mathematical algorithms are the algorithm of Myers and Miller (1988) *CABIOS 4*:11-17; the local homology algorithm of Smith *et al.* (1981) *Adv. Appl. Math. 2*:482; the

20   homology alignment algorithm of Needleman and Wunsch (1970) *J. Mol. Biol. 48*:443-453; the search-for-similarity-method of Pearson and Lipman (1988) *Proc. Natl. Acad. Sci. 85*:2444-2448; the algorithm of Karlin and Altschul (1990) *Proc. Natl. Acad. Sci. USA 872264*, modified as in Karlin and Altschul (1993) *Proc. Natl. Acad. Sci. USA 90*:5873-5877.

25   Computer implementations of these mathematical algorithms can be utilized for comparison of sequences to determine sequence identity. Such implementations include, but are not limited to: CLUSTAL in the PC/Gene program (available from Intelligenetics, Mountain View, California); the ALIGN program (Version 2.0) and GAP, BESTFIT, BLAST, FASTA, and TFASTA in the Wisconsin Genetics Software Package,

30   Version 10 (available from Genetics Computer Group (GCG), 575 Science Drive,

Madison, Wisconsin, USA). Alignments using these programs can be performed using the default parameters. The CLUSTAL program is well described by Higgins *et al.* (1988) *Gene 73*:237-244 (1988); Higgins *et al.* (1989) *CABIOS 5*:151-153; Corpet *et al.* (1988) *Nucleic Acids Res. 16*:10881-90; Huang *et al.* (1992) *CABIOS 8*:155-65; and

5   Pearson *et al.* (1994) *Meth. Mol. Biol. 24*:307-331. The ALIGN program is based on the algorithm of Myers and Miller (1988) *supra*. A PAM120 weight residue table, a gap length penalty of 12, and a gap penalty of 4 can be used with the ALIGN program when comparing amino acid sequences. The BLAST programs of Altschul *et al* (1990) *J. Mol. Biol. 215*:403 are based on the algorithm of Karlin and Altschul (1990) *supra*. BLAST

10   nucleotide searches can be performed with the BLASTN program, score = 100, wordlength = 12, to obtain nucleotide sequences homologous to a nucleotide sequence encoding a protein of the invention. BLAST protein searches can be performed with the BLASTX program, score = 50, wordlength = 3, to obtain amino acid sequences homologous to a protein or polypeptide of the invention. To obtain gapped alignments

15   for comparison purposes, Gapped BLAST (in BLAST 2.0) can be utilized as described in Altschul *et al.* (1997) *Nucleic Acids Res. 25*:3389. Alternatively, PSI-BLAST (in BLAST 2.0) can be used to perform an iterated search that detects distant relationships between molecules. See Altschul *et al.* (1997) *supra*. When utilizing BLAST, Gapped BLAST, PSI-BLAST, the default parameters of the respective programs (e.g., BLASTN for

20   nucleotide sequences, BLASTX for proteins) can be used. See http://www.ncbi.nlm.nih.gov. Alignment may also be performed manually by inspection.

Unless otherwise stated, sequence identity/similarity values provided herein refer to the value obtained using GAP version 10 using the following parameters: % identity using GAP Weight of 50 and Length Weight of 3; % similarity using Gap Weight of 12

25   and Length Weight of 4, or any equivalent program. By "equivalent program" is intended any sequence comparison program that, for any two sequences in question, generates a global alignment having identical nucleotide or amino acid residue matches and an identical percent sequence identity when compared to the corresponding alignment generated by GAP Version 10.

GAP uses the algorithm of Needleman and Wunsch (1970) *J. Mol. Biol.* 48: 443-453, to find the alignment of two complete sequences that maximizes the number of matches and minimizes the number of gaps. GAP considers all possible alignments and gap positions and creates the alignment with the largest number of matched bases and the

5   fewest gaps. It allows for the provision of a gap creation penalty and a gap extension penalty in units of matched bases. GAP must make a profit of gap creation penalty number of matches for each gap it inserts. If a gap extension penalty greater than zero is chosen, GAP must, in addition, make a profit for each gap inserted of the length of the gap times the gap extension penalty. Default gap creation penalty values and gap

10   extension penalty values in Version 10 of the Wisconsin Genetics Software Package for protein sequences are 8 and 2, respectively. For nucleotide sequences the default gap creation penalty is 50 while the default gap extension penalty is 3. The gap creation and gap extension penalties can be expressed as an integer selected from the group of integers consisting of from 0 to 200. Thus, for example, the gap creation and gap extension

15   penalties can be 0, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 15, 20, 25, 30, 35, 40, 45, 50, 55, 60, 65 or greater.

GAP presents one member of the family of best alignments. There may be many members of this family, but no other member has a better quality. GAP displays four figures of merit for alignments: Quality, Ratio, Identity, and Similarity. The Quality is

20   the metric maximized in order to align the sequences. Ratio is the quality divided by the number of bases in the shorter segment. Percent Identity is the percent of the symbols that actually match. Percent Similarity is the percent of the symbols that are similar. Symbols that are across from gaps are ignored. A similarity is scored when the scoring matrix value for a pair of symbols is greater than or equal to 0.50, the similarity

25   threshold. The scoring matrix used in Version 10 of the Wisconsin Genetics Software Package is BLOSUM62 for polypeptides and NWSEAPDNA for polynucleotides (see Henikoff and Henikoff (1989) *Proc. Natl. Acad. Sci. USA* 89:10915).

(c)   As used herein, "sequence identity" or "identity" in the context of two nucleic acid or polypeptide sequences makes reference to the residues in the two

30   sequences that are the same when aligned for maximum correspondence over a specified

comparison window. When percentage of sequence identity is used in reference to proteins it is recognized that residue positions which are not identical often differ by conservative amino acid substitutions, where amino acid residues are substituted for other amino acid residues with similar chemical properties (e.g., charge or hydrophobicity) and

5   therefore do not change the functional properties of the molecule. When sequences differ in conservative substitutions, the percent sequence identity may be adjusted upwards to correct for the conservative nature of the substitution. Sequences that differ by such conservative substitutions are said to have "sequence similarity" or "similarity". Means for making this adjustment are well known to those of skill in the art. Typically this

10   involves scoring a conservative substitution as a partial rather than a full mismatch, thereby increasing the percentage sequence identity. Thus, for example, where an identical amino acid is given a score of 1 and a non-conservative substitution is given a score of zero, a conservative substitution is given a score between zero and 1. The scoring of conservative substitutions is calculated, e.g., as implemented in the program

15   PC/GENE (Intelligenetics, Mountain View, California).

(d)   As used herein, "percentage of sequence identity" means the value determined by comparing two optimally aligned sequences over a comparison window, wherein the portion of the polynucleotide or polypeptide sequence in the comparison window may comprise additions or deletions (i.e., gaps) as compared to the reference

20   sequence (which does not comprise additions or deletions) for optimal alignment of the two sequences. The percentage is calculated by determining the number of positions at which the identical nucleic acid base or amino acid residue occurs in both sequences to yield the number of matched positions, dividing the number of matched positions by the total number of positions in the window of comparison, and multiplying the result by 100

25   to yield the percentage of sequence identity.

(e)(i)   The term "substantial identity" of polynucleotide sequences means that a polynucleotide comprises a sequence that has at least 70% sequence identity, preferably at least 80%, more preferably at least 90%, and most preferably at least 95%, compared to a reference sequence using one of the alignment programs described using standard

30   parameters. One of skill in the art will recognize that these values can be appropriately

adjusted to determine corresponding identity of proteins encoded by two nucleotide sequences by taking into account codon degeneracy, amino acid similarity, reading frame positioning, and the like. Substantial identity of amino acid sequences for these purposes normally means sequence identity of at least 60%, more preferably at least 70%, 80%,

5   90%, and most preferably at least 95%.

Another indication that nucleotide sequences are substantially identical is if two molecules hybridize to each other under stringent conditions. Generally, stringent conditions are selected to be about 5°C lower than the thermal melting point (T_m) for the specific sequence at a defined ionic strength and pH. However, stringent conditions

10   encompass temperatures in the range of about 1°C to about 20°C lower than the T_m, depending upon the desired degree of stringency as otherwise qualified herein. Nucleic acids that do not hybridize to each other under stringent conditions are still substantially identical if the polypeptides they encode are substantially identical. This may occur, e.g., when a copy of a nucleic acid is created using the maximum codon degeneracy permitted

15   by the genetic code. One indication that two nucleic acid sequences are substantially identical is when the polypeptide encoded by the first nucleic acid is immunologically cross reactive with the polypeptide encoded by the second nucleic acid.

(e)(ii) The term "substantial identity" in the context of a peptide indicates that a peptide comprises a sequence with at least 70% sequence identity to a reference

20   sequence, preferably 80%, more preferably 85%, most preferably at least 90% or 95% sequence identity to the reference sequence over a specified comparison window. Preferably, optimal alignment is conducted using the homology alignment algorithm of Needleman and Wunsch (1970) *J. Mol. Biol. 48*:443-453. An indication that two peptide sequences are substantially identical is that one peptide is immunologically reactive with

25   antibodies raised against the second peptide. Thus, a peptide is substantially identical to a second peptide, for example, where the two peptides differ only by a conservative substitution. Peptides that are "substantially similar" share sequences as noted above except that residue positions that are not identical may differ by conservative amino acid changes.

The *MSH2* nucleotide sequences find use in methods for altering DNA repair processes in plants and other organisms. MSH2 is known to be involved in mismatch-repair systems of organisms. Furthermore, organisms harboring mutant alleles of *msh2* display increased levels of recombination relative to wild-type organisms (deWind *et al.*

5   (1995) *Cell 82*:321-330). While the present invention does not depend on a particular mechanism, it is believed that MSH2 facilitates mismatch repair through direct interactions with several other proteins, including, but not limited to, MSH3 and MSH6. To alter mismatch repair in an organism, the organism can be transformed with an *MSH2* nucleotide sequence of the invention, or a fragment or variant thereof.

10   An alteration in mismatch repair in an organism can comprise at least one of change in the DNA of an organism, or at least one cell thereof. Such changes include, but are not limited to, substitutions, additions, deletions, inversions, and other rearrangements. Typically, such an alteration in mismatch repair can be determined by monitoring mutation frequency. Methods for monitoring mutation frequency are known

15   in the art and typically involve determining whether a change has occurred in the DNA sequence of one or more genes by monitoring loss, or gain, of a particular function associated with a particular product encoded by the gene. Other methods can be employed, however, to ascertain mutation frequency at the nucleic acid level including, but not limited to, RFLP analysis, PCR, and DNA sequencing. Typically, mutation

20   frequency is assessed by comparing the mutation frequency of an organism that is modified according to the methods of the present invention to a control organism or similar unmodified organism.

The methods of the invention additionally find use in altering recombination frequency in plants and other organisms. By expressing an MSH2 nucleotide sequence of

25   the invention in a plant or other organism, recombination efficiency can be altered. While the invention does not depend on a particular biological mechanism, MSH2 is believed to be involved in the resolution of the Holliday junction that occurs during genetic recombination *in vivo*. Decreasing the level or activity of MSH2 in an organism is expected to increase the integration of exogenous DNA through homologous or

30   homeologous recombination into specific targets within the genome. In a mouse *msh2*

cell line, target integration of plasmid DNA at the Rb locus was increased about fifty-fold (deWind *et al.* (1995) *Cell 82*:321-330). Thus, the MSH2 nucleotide sequences can be used to increase integration of foreign DNA into target genes within the genome. Furthermore, the MSH2 nucleotide sequences can be employed to increase the efficiency

5 of methods of *in vivo* genetic modification. Such methods are believed to involve recombination, and include, for example, chimeraplasty and gene replacement.

By "exogenous DNA" is intended any nucleic acid molecule that is introduced into a cell. It is recognized that the invention also encompasses nucleic acid molecules comprised of deoxyribonucleotides, ribonucleotides, and combination thereof. Such

10 deoxyribonucleotides and ribonucleotides include, but not limited to, naturally occurring and synthetic form, and derivatives thereof.

By lowering the level or activity of MSH2 in a plant or other organism, the efficiency of chimeraplasty or gene replacement can be increased. By "efficiency of chimeraplasty" or "efficiency of gene replacement" is intended the proportion of cells or

15 organisms having the desired genetic modification recovered from the total number of cells or organisms used in a chimeraplasty or gene replacement attempt, respectively.

The methods of the invention additionally encompass the use of dominant-negative strategies to reduce a particular biological activity of a MutS homologue or MSH2 within an organism. Such strategies involve the expression in an organism of an

20 *MSH2* nucleotide sequence of the invention, or fragment thereof that encodes a portion of the MSH2. The methods of the invention additionally encompass nucleotide sequences encoding variants of the MSH2 proteins of the invention, and fragments thereof, that can be used in dominant-negative strategies to reduce the biological activity of a MutS homologue or MSH2 within an organism or cell thereof. Such dominant-negative

25 strategies are known in the art and can involve the expression of a modified subunit of a multisubunit protein. See, for example, Alani *et al.* (1997) *Mol. Cell Biol. 17*:2436-244; Drotschmann *et al.* (1999) *Proc. Natl. Acad. Sci. USA 96*:2970-2975; and Wu and Marinus (1994) *J. Bact. 176*: 5393-5400; all of which are hereby herein incorporated by reference. Generally, such a modified subunit comprises a polypeptide that is able to

30 affect, or interact with, other members of the multisubunit protein complex and thereby

reduce, or eliminate, the biological activity of the complex. While the methods of the invention do not depend a particular biological mechanism, typically such a dominant-negative approach will involve the expression of a variant of a MSH2 protein of the invention that does not possess the complete biological activity of the native protein. It is recognized that such an dominant-negative approach does not depend on eliminating or reducing the expression of native *MSH2* genes in a plant, only that such an approach involves the expression of a variant of an MSH2 of the invention that is capable of causing a dominant-negative phenotype.

By "dominant-negative phenotype" is intended a phenotype that, when compared to a wild type phenotype or a previous phenotype of the organism, is substantially altered in a negative manner including, but not limited to, a loss or reduction in a particularly cellular function such as, for example, an enzyme activity or mismatch repair. Further it is recognized that, while the methods of the invention can be used to negatively affect, through a dominant-negative approach, the cellular activity of an MSH2 protein, or complex thereof, desired phenotypic changes can result in a organism including, but not limited to, an increase in recombination, an improvement in transformation efficiency and an increase in the efficiency of chimeraplasty.

In an embodiment of the invention, a nucleotide construct comprising an *MSH2* sequence of the invention, or variant or fragment thereof, is introduced into an organism or host cell, particularly a bacterial cell, more particularly an *E. coli* cell. DNA repair is then monitored in the transformed organism, or host cell, by, for example, determining the mutation rate. Such transformed organisms and host cells find use in producing *MSH2* nucleotide sequences that can be used in dominant-negative strategies to disrupt DNA repair processes in bacteria, plants, and other organisms. Such desired *MSH2* nucleotide sequences encode dominant-negative MSH2 variants. By "dominant-negative MSH2 variant" is intended a polypeptide that is capable of conferring a dominant-negative phenotype on a host cell. In particular, the dominant-negative phenotype will impair DNA repair in a host cell or organism. Such an impairment in DNA repair can cause an increase in the mutation rate and/or recombination frequency in a host cell or organism. Thus, the desired *MSH2* nucleotide sequence, which encodes a dominant-

negative MSH2 variant, can be identified by, for example, selecting a host cell with impaired DNA repair as detected by an increase in the mutation rate and/or recombination frequency therein. Such desired *MSH2* nucleotide sequences and the dominant-negative MSH2 variants encoded thereby find use in methods for altering DNA

5   repair processes, particularly methods for increasing the mutation and/or recombination rates in an organism.

Thus, the invention provides methods for identifying *MSH2* nucleotide sequences which encode dominant-negative MSH2 variants that are capable of conferring a dominant-negative phenotype on a cell. The invention further provides isolated *MSH2*

10   nucleotide sequences encoding such MSH2 variants, the dominant-negative MSH2 variants encoded thereby, and host cells and organisms transformed with such *MSH2* nucleotide sequences. Such transformed host cells and organisms include, but are not limited to, bacteria, yeast, fungi, animals and plants.

The dominant-negative MSH2 variant of the invention involve the use of an

15   MSH2 amino acid sequence having at least one amino acid substitution, truncation, internal deletion or insertion. Any MSH2 nucleotide sequence and any MSH2 amino acid sequence known the art can be used in the methods of the present invention. Such *MSH2* nucleotide sequences and amino acid sequences include, but are not limited to, GenBank Accession Nos. AF109243, AF003005, AF002706, AF026549, U87911, and

20   M84170. At least one substitution , truncation, internal deletion or insertion can be introduced in the amino acid sequence of an MSH2 protein by, for example, modifying the nucleotide sequence that encodes the MSH2 protein using methods known in the art. The modified *MSH2* nucleotide sequence can then be introduced into an organism or host cell according to the methods of the present invention.

25   For expression in *E. coli*, the expression cassette can additionally comprise an operably linked promoter. Preferably, such a promoter drives high level gene expression in *E. coli*, such as, for example, the T5 promoter. The expression cassette can further comprise a nucleotide sequence that encodes an epitope or tag that can be readily detected by immunological or other known methods. Such epitopes or tags, and methods

30   for their use, are known in the art. A nucleotide sequence encoding the epitope or tag can

be operably linked to the *MSH2* nucleotide sequence for the transcription of a fusion protein comprising the MSH2 amino acid sequence and the amino acid sequence of the epitope or tag. Typically, the epitope or tag is N-terminal or C-terminal relative to the MSH2 amino acid sequence. Such epitopes and tags are known in the art to be useful for

5   the detection and/or purification of fusion proteins.

In another embodiment of the invention, methods are provided for decreasing the level or activity of an MSH2 protein of the invention in a plant or cell thereof. Plants or cells with decreased MSH2 protein or activity find use in methods for increasing recombination frequency, increasing mutation rate and increasing the efficiency of

10   chimeraplasty. The level or activity of MSH2 can be reduced in the plant or cell by, for example, introducing into the plant or cell, a nucleotide construct comprising a promoter that drives expression in a plant operably linked to an *MSH2* nucleotide sequence of the invention. The methods can additionally involve co-suppression, antisense suppression or a dominant-negative strategy to reduce or substantially eliminate the biological activity

15   of MSH2.

Alternatively, an *MSH2* nucleotide sequence of the invention that encodes an MSH2 protein that is known to cause a dominant-negative phenotype, can be directly introduced into a plant or other host cell. Such MSH2 proteins encompass the fragments and variants as discussed supra. Any method for introducing a protein into a plant or

20   other host cell that is known in the art can be employed in the methods of the present invention. For example, a protein can be introduced into a plant by particle bombardment in a manner analogous to that used for the introduction of nucleic acids. *See*, U.S. Patent No. 4,945.050. The MSH2 protein can be associated with or precipitated onto the microprojectiles or microparticles and then bombarded into plant cells. Nucleotide

25   constructs comprising, for example, a chimeraplast or other nucleotide sequence comprising a gene of interest can also be associated with the same microprojectiles or two separate groups of microprojectiles—one with the MSH2 protein and the other with the chimeraplast or nucleotide sequence of interest—can be prepared and then co-bombarded. Alternatively, the plant cells can be bombarded separately with the MSH2-

associated particles and the chimeraplast-associated or nucleotide construct-associated particles.

In another embodiment of the invention, the MSH2 protein can be produced in an *Agrobacterium* cell and delivered to a plant cell by the *Agrobacterium* cell at about the same time as the bacterial cell delivers its Ti plasmid, comprising a gene of interest, to the plant cell. To produce an MSH2 protein of the invention in *Agrobacterium*, an *Agrobacterium* cell can be transformed with an MSH2 nucleotide sequence of the invention that is operably linked to a promoter that drives expression in the bacterial cell. Methods for transforming *Agrobacterium* are known in the art and include, but are not limited to, electroporation. Promoters that drive the expression of operably linked nucleotide sequences in *Agrobacterium* are also known in the art. It is recognized that to facilitate the transfer of the MSH2 protein into a plant cell, fusion proteins comprising at least a portion of an MSH2 protein of the invention and at least a portion of one or more additional proteins such as, for example, VirF and VirE2 can be obtained by preparing a nucleotide construct comprising at least a portion of an *MSH2* nucleotide sequence of the invention operable operably linked to a coding sequence for the additional protein or desired portion thereof. The construction of such fusion proteins for transfer by *Agrobacterium* to a plant cell, and methods of their use, are known to those of ordinary skill in the art. Generally, such methods involve fusing the protein of interest to the N-terminal end of a VirF, VirE2, or a transport domain thereof. The transport domains of VirF and VirE2 proteins are known to be located in the C-terminal regions of the proteins. See, Vergunst *et al.* ((2000) Science 290:979-982) and the references cited therein; herein incorporated by reference.

The *MSH2* nucleotide sequences and proteins of the invention find further use in methods for improving transformation efficiency. By "improving transformation efficiency" is intended an increase in the recovery of transformed cells, tissues, organs, or organisms from a transformation attempt. The methods of the invention involve introducing one or more MSH2 nucleotide sequences or proteins into a host cell. Such host cells can provide improved transformation efficiency in a transformation attempt. Typically, the *Msh* nucleotide sequences or proteins will be introduced into a host cell

prior to, or concomitantly, with a nucleotide sequence of interest to improve transformation efficiency with respect to the nucleotide sequence of interest. In particular, the methods can increase the recovery of stably transformed cells, tissues, organs, or organisms in a transformation attempt. Thus, the invention further provides

5   improved methods for transforming organisms, particularly plants.

While invention does not depend on a particularly biological or genetic mechanism, it is recognized that altering DNA mismatch repair and DNA recombination in an organism can affect cellular processes that are involved in the stable incorporation of a nucleotide construct of interest, or at least one nucleotide thereof, into the genome of

10   the cell. Further, it is recognized that reducing, or otherwise inhibiting, DNA mismatch repair in an organism can improve the efficiency of genetic transformation methods, such as, for example, chimeraplasty, which are believed to involve circumventing the DNA mismatch-repair system of a host cell. Similarly, is also recognized that reducing, or otherwise inhibiting, the activity or function of a protein that is known to prevent or

15   negatively impact recombination in a cell can increase recombination in the cell. Yeast *msh2* mutants have decreased mismatch repair and increased recombination, particularly recombination between partially homologous (homeologous) sequences (Alani *et al.* (1994) *Genetics 137*: 9-39.)

In an embodiment of the invention, a plant is stably transformed with a nucleotide

20   construct comprising an *MSH2* nucleotide sequence of the invention operably linked to a promoter that drives expression in a plant cell. Such a plant finds use in methods for improving transformation efficiency. Preferably, the promoter drives expression in plant cells that are targeted for transformation with a nucleotide sequence of interest. More preferably, the promoter is a tissue-preferred or chemical-regulated promoter.

25   The invention additionally provides nucleotide sequences of *MSH2* promoters. The *MSH2* promoter nucleotide sequences of the invention find use in methods for regulating gene expression in a plant. Such methods involve operably linking an MSH2 promoter sequence of the invention to a heterologous nucleotide sequence wherein the *MSH2* promoter allows the transcription of the heterologous nucleotide sequence. By

30   "heterologous nucleotide sequence" is intended any nucleotide sequence that is not

identical to that entire portion of the native MSH2 gene that immediately follows, in the 3' direction, the MSH2 promoter of the invention, as is found within the native MSH2 gene from which the promoter originates. While the invention does not depend on a particular heterologous nucleotide sequence, preferred sequences are coding sequences.

5   The heterologous nucleotide can be operably linked in either the sense or antisense orientation depending on the desired outcome.

The *MSH2* sequences of the invention are provided in expression cassettes for expression in the plant or other organism of interest. For *MSH2* coding sequences, the cassette will include 5' and 3' regulatory sequences operably linked to a *MSH2* sequence

10   of the invention. By "operably linked" is intended a functional linkage between a promoter and a second sequence, wherein the promoter sequence initiates and mediates transcription of the DNA sequence corresponding to the second sequence. Generally, operably linked means that the nucleic acid sequences being linked are contiguous and, where necessary to join two protein coding regions, contiguous and in the same reading

15   frame. The cassette may additionally contain at least one additional gene to be cotransformed into the organism. Alternatively, the additional gene(s) can be provided on multiple expression cassettes.

The expression cassettes are provided with a plurality of restriction sites for insertion of the *MSH2* coding sequence to be under the transcriptional regulation of the

20   regulatory regions. The expression cassette may additionally contain selectable marker genes.

The expression cassette will include in the 5'-3' direction of transcription, a transcriptional and translational initiation region, a *MSH2* DNA sequence of the invention, and a transcriptional and translational termination region functional in plants.

25   The transcriptional initiation region, the promoter, may be native or analogous or foreign or heterologous to the plant host. Additionally, the promoter may be the natural sequence or alternatively a synthetic sequence. By "foreign" is intended that the transcriptional initiation region is not found in the native plant into which the transcriptional initiation region is introduced. As used herein, a "chimeric gene" comprises a coding sequence

30   operably linked to a transcription initiation region that is heterologous to the coding

sequence, or any combination of a promoter with a coding sequence that is not identical to the structure of a native, unmodified gene.

While it may be preferable to express the sequences using heterologous promoters, the native promoter sequences may be used. Such constructs would change
5 expression levels of MSH2 in the plant or plant cell. Thus, the phenotype of the plant or plant cell is altered.

The termination region may be native with the transcriptional initiation region, may be native with the operably linked DNA sequence of interest, or may be derived from another source. Convenient termination regions are available from the Ti-plasmid
10 of *A. tumefaciens*, such as the octopine synthase and nopaline synthase termination regions. See also Guerineau *et al.* (1991) *Mol. Gen. Genet. 262*:141-144; Proudfoot (1991) *Cell 64*:671-674; Sanfacon *et al.* (1991) *Genes Dev. 5*:141-149; Mogen *et al.* (1990) *Plant Cell 2*:1261-1272; Munroe *et al.* (1990) *Gene 91*:151-158; Ballas *et al.* (1989) *Nucleic Acids Res. 17*:7891-7903; and Joshi *et al.* (1987) *Nucleic Acid Res.*
15 *15*:9627-9639.

Similarly, the *MSH2* promoter sequences of the invention are provided in expression cassettes for expression in the plant of interest. The cassette will include an *MSH2* promoter sequence of the invention operably linked to a coding sequence. Any coding sequence known in the art can be used. The cassette will additionally comprise 3'
20 regulatory sequences. If necessary, the cassette can also contain additionally 5' regulatory sequences.

Where appropriate, the gene(s) may be optimized for increased expression in the transformed plant. That is, the genes can be synthesized using plant-preferred codons for improved expression. See, for example, Campbell and Gowri (1990) *Plant Physiol.* 92:1-
25 11 for a discussion of host-preferred codon usage. Methods are available in the art for synthesizing plant-preferred genes. See, for example, U.S. Patent Nos. 5,380,831, and 5,436,391, and Murray *et al.* (1989) *Nucleic Acids Res. 17*:477-498, herein incorporated by reference.

Additional sequence modifications are known to enhance gene expression in a
30 cellular host. These include elimination of sequences encoding spurious polyadenylation

signals, exon-intron splice site signals, transposon-like repeats, and other such well-characterized sequences that may be deleterious to gene expression. The G-C content of the sequence may be adjusted to levels average for a given cellular host, as calculated by reference to known genes expressed in the host cell. When possible, the sequence is

5   modified to avoid predicted hairpin secondary mRNA structures.

The expression cassettes may additionally contain 5' leader sequences in the expression cassette construct. Such leader sequences can act to enhance translation. Translation leaders are known in the art and include: picornavirus leaders, for example, EMCV leader (Encephalomyocarditis 5' noncoding region) (Elroy-Stein *et al.* (1989)

10   *Proc. Natl. Acad. Sci. USA 86*:6126-6130); potyvirus leaders, for example, TEV leader (Tobacco Etch Virus) (Gallie *et al.* (1995) *Gene 165(2)*:233-238), MDMV leader (Maize Dwarf Mosaic Virus) (*Virology 154*:9-20), and human immunoglobulin heavy-chain binding protein (BiP) (Macejak *et al.* (1991) *Nature 353*:90-94); untranslated leader from the coat protein mRNA of alfalfa mosaic virus (AMV RNA 4) (Jobling *et al.* (1987)

15   *Nature 325*:622-625); tobacco mosaic virus leader (TMV) (Gallie *et al.* (1989) in *Molecular Biology of RNA*, ed. Cech (Liss, New York), pp. 237-256); and maize chlorotic mottle virus leader (MCMV) (Lommel *et al.* (1991) *Virology 81*:382-385). See also, Della-Cioppa *et al.* (1987) *Plant Physiol. 84*:965-968. Other methods known to enhance translation can also be utilized, for example, introns, and the like.

20   In preparing the expression cassette, the various DNA fragments may be manipulated, so as to provide for the DNA sequences in the proper orientation and, as appropriate, in the proper reading frame. Toward this end, adapters or linkers may be employed to join the DNA fragments or other manipulations may be involved to provide for convenient restriction sites, removal of superfluous DNA, removal of restriction sites,

25   or the like. For this purpose, *in vitro* mutagenesis, primer repair, restriction, annealing, resubstitutions, e.g., transitions and transversions, may be involved.

A number of promoters can be used in the practice of the invention. The promoters can be selected based on the desired outcome. The nucleic acids can be combined with constitutive, tissue-preferred, or other promoters for expression in plants.

Such constitutive promoters include, for example, the core promoter of the Rsyn7 promoter and other constitutive promoters disclosed in WO 99/43838 and U.S. Patent No. 6,072,050; the core CaMV 35S promoter (Odell *et al.* (1985) *Nature* 313:810-812); rice actin (McElroy *et al.* (1990) *Plant Cell* 2:163-171); ubiquitin (Christensen *et al.*

5   (1989) *Plant Mol. Biol.* 12:619-632 and Christensen *et al.* (1992) *Plant Mol. Biol.* 18:675-689); pEMU (Last *et al.* (1991) *Theor. Appl. Genet.* 81:581-588); MAS (Velten *et al.* (1984) *EMBO J.* 3:2723-2730); ALS promoter (U.S. Patent No. 5,659,026), and the like. Other constitutive promoters include, for example, U.S. Patent Nos. 5,608,149; 5,608,144; 5,604,121; 5,569,597; 5,466,785; 5,399,680; 5,268,463; 5,608,142; and

10   6,177,611.

Chemical-regulated promoters can be used to modulate the expression of a gene in a plant through the application of an exogenous chemical regulator. Depending upon the objective, the promoter may be a chemical-inducible promoter, where application of the chemical induces gene expression, or a chemical-repressible promoter, where

15   application of the chemical represses gene expression. Chemical-inducible promoters are known in the art and include, but are not limited to, the maize In2-2 promoter, which is activated by benzenesulfonamide herbicide safeners, the maize GST promoter, which is activated by hydrophobic electrophilic compounds that are used as pre-emergent herbicides, and the tobacco PR-1a promoter, which is activated by salicylic acid. Other

20   chemical-regulated promoters of interest include steroid-responsive promoters (see, for example, the glucocorticoid-inducible promoter in Schena *et al.* (1991) *Proc. Natl. Acad. Sci. USA* 88:10421-10425 and McNellis *et al.* (1998) *Plant J.* 14(2):247-257) and tetracycline-inducible and tetracycline-repressible promoters (see, for example, Gatz *et al.* (1991) *Mol. Gen. Genet.* 227:229-237, and U.S. Patent Nos. 5,814,618 and

25   5,789,156), herein incorporated by reference.

Tissue-preferred promoters can be utilized to target enhanced *MSH2* expression within a particular plant tissue. Tissue-preferred promoters include, but are not limited to, those described by Yamamoto *et al.* (1997) *Plant J.* 12(2)255-265; Kawamata *et al.* (1997) *Plant Cell Physiol.* 38(7):792-803; Hansen *et al.* (1997) *Mol. Gen Genet.*

30   254(3):337-343; Russell *et al.* (1997) *Transgenic Res.* 6(2):157-168; Rinehart *et al.*

(1996) *Plant Physiol. 112(3)*:1331-1341; Van Camp *et al.* (1996) *Plant Physiol. 112(2)*:525-535; Canevascini *et al.* (1996) *Plant Physiol. 112(2)*:513-524; Yamamoto *et al.* (1994) *Plant Cell Physiol. 35(5)*:773-778; Lam (1994) *Results Probl. Cell Differ.* *20*:181-196; Orozco *et al.* (1993) *Plant Mol Biol. 23(6)*:1129-1138; Matsuoka *et al.*

5   (1993) *Proc Natl. Acad. Sci. USA 90(20)*:9586-9590; and Guevara-Garcia *et al.* (1993) *Plant J. 4(3)*:495-505. Such promoters can be modified, if necessary, for weak expression.

Generally, the expression cassette will comprise a selectable marker gene for the selection of transformed cells. Selectable marker genes are utilized for the selection of

10   transformed cells or tissues. Marker genes include genes encoding antibiotic resistance, such as those encoding neomycin phosphotransferase II (NEO) and hygromycin phosphotransferase (HPT), as well as genes conferring resistance to herbicidal compounds, such as glufosinate ammonium, bromoxynil, imidazolinones, and 2,4-dichlorophenoxyacetate (2,4-D). See generally, Yarranton (1992) *Curr. Opin. Biotech.*

15   *3*:506-511; Christopherson *et al.* (1992) *Proc. Natl. Acad. Sci. USA 89*:6314-6318; Yao *et al.* (1992) *Cell 71*:63-72; Reznikoff (1992) *Mol. Microbiol. 6*:2419-2422; Barkley *et al.* (1980) in *The Operon*, pp. 177-220; Hu *et al.* (1987) *Cell 48*:555-566; Brown *et al.* (1987) *Cell 49*:603-612; Figge *et al.* (1988) *Cell 52*:713-722; Deuschle *et al.* (1989) *Proc. Natl. Acad. Aci. USA 86*:5400-5404; Fuerst *et al.* (1989) *Proc. Natl. Acad. Sci. USA 86*:2549-

20   2553; Deuschle *et al.* (1990) *Science 248*:480-483; Gossen (1993) Ph.D. Thesis, University of Heidelberg; Reines *et al.* (1993) *Proc. Natl. Acad. Sci. USA 90*:1917-1921; Labow *et al.* (1990) *Mol. Cell. Biol. 10*:3343-3356; Zambretti *et al.* (1992) *Proc. Natl. Acad. Sci. USA 89*:3952-3956; Baim *et al.* (1991) *Proc. Natl. Acad. Sci. USA 88*:5072-5076; Wyborski *et al.* (1991) *Nucleic Acids Res. 19*:4647-4653; Hillenand-Wissman (1989) *Topics Mol. Struc.*

25   *Biol. 10*:143-162; Degenkolb *et al.* (1991) *Antimicrob. Agents Chemother. 35*:1591-1595; Kleinschnidt *et al.* (1988) *Biochemistry 27*:1094-1104; Bonin (1993) Ph.D. Thesis, University of Heidelberg; Gossen *et al.* (1992) *Proc. Natl. Acad. Sci. USA 89*:5547-5551; Oliva *et al.* (1992) *Antimicrob. Agents Chemother. 36*:913-919; Hlavka *et al.* (1985) *Handbook of Experimental Pharmacology*, Vol. 78 (Springer-Verlag, Berlin); Gill *et al.*

30   (1988) *Nature 334*:721-724. Such disclosures are herein incorporated by reference.

The above list of selectable marker genes is not meant to be limiting. Any selectable marker gene can be used in the present invention.

Transformation protocols as well as protocols for introducing nucleotide sequences into plants may vary depending on the type of plant or plant cell, i.e., monocot

5   or dicot, targeted for transformation. Suitable methods of introducing nucleotide sequences into plant cells and subsequent insertion into the plant genome include microinjection (Crossway *et al.* (1986) *Biotechniques* 4:320-334), electroporation (Riggs *et al.* (1986) *Proc. Natl. Acad. Sci. USA* 83:5602-5606, *Agrobacterium*-mediated transformation (Townsend *et al.*, U.S. Patent No. 5,563,055; Zhao *et al.*, U.S. Patent No.

10   5,981,840), direct gene transfer (Paszkowski *et al.* (1984) *EMBO J.* 3:2717-2722), and ballistic particle acceleration (see, for example, Sanford *et al.*, U.S. Patent No. 4,945,050; Tomes *et al.*, U.S. Patent No. 5,879,918; Tomes *et al.*, U.S. Patent No. 5,886,244; Bidney *et al.*, U.S. Patent No. 5,932,782; Tomes *et al.* (1995) "Direct DNA Transfer into Intact Plant Cells via Microprojectile Bombardment," in *Plant Cell, Tissue, and Organ Culture:*

15   *Fundamental Methods*, ed. Gamborg and Phillips (Springer-Verlag, Berlin); *McCabe et al.* (1988) *Biotechnology* 6:923-926); and Lec1 transformation (WO 00/28058). Also see Weissinger *et al.* (1988) *Annu. Rev. Genet.* 22:421-477; Sanford *et al.* (1987) *Particulate Science and Technology* 5:27-37 (onion); Christou *et al.* (1988) *Plant Physiol.* 87:671-674 (soybean); McCabe *et al.* (1988) *Bio/Technology* 6:923-926 (soybean); Finer

20   and McMullen (1991) *In Vitro Cell Dev. Biol.* 27P:175-182 (soybean); Singh *et al.* (1998) *Theor. Appl. Genet.* 96:319-324 (soybean); Datta *et al.* (1990) *Biotechnology* 8:736-740 (rice); Klein *et al.* (1988) *Proc. Natl. Acad. Sci. USA* 85:4305-4309 (maize); Klein *et al.* (1988) *Biotechnology* 6:559-563 (maize); Tomes, U.S. Patent No. 5,240,855; Buising *et al.*, U.S. Patent Nos. 5,322,783 and 5,324,646; Tomes *et al. (1995)* "Direct

25   DNA Transfer into Intact Plant Cells via Microprojectile Bombardment," in *Plant Cell, Tissue, and Organ Culture: Fundamental Methods*, ed. Gamborg (Springer-Verlag, Berlin) (maize); Klein *et al.* (1988) *Plant Physiol.* 91:440-444 (maize); Fromm *et al.* (1990) *Biotechnology* 8:833-839 (maize); Hooykaas-Van Slogteren *et al.* (1984) *Nature (London)* 311:763-764; Bowen *et al.*, U.S. Patent No. 5,736,369 (cereals); Bytebier *et al.*

30   (1987) *Proc. Natl. Acad. Sci. USA* 84:5345-5349 (Liliaceae); De Wet *et al.* (1985) in *The*

Experimental Manipulation of Ovule Tissues, ed. Chapman *et al.* (Longman, New York), pp. 197-209 (pollen); Kaeppler *et al.* (1990) *Plant Cell Reports 9*:415-418 and Kaeppler *et al.* (1992) *Theor. Appl. Genet. 84*:560-566 (whisker-mediated transformation); D'Halluin *et al.* (1992) *Plant Cell 4*:1495-1505 (electroporation); Li *et al.* (1993) *Plant*

5 *Cell Reports 12*:250-255 and Christou and Ford (1995) *Annals of Botany 75*:407-413 (rice); Osjoda *et al.* (1996) *Nature Biotechnology 14*:745-750 (maize via *Agrobacterium tumefaciens*); all of which are herein incorporated by reference.

The cells that have been transformed may be grown into plants in accordance with conventional ways. See, for example, McCormick *et al.* (1986) *Plant Cell Reports 5*:81-

10 84. These plants may then be grown, and either pollinated with the same transformed strain or different strains, and the resulting hybrid having constitutive expression of the desired phenotypic characteristic identified. Two or more generations may be grown to ensure that expression of the desired phenotypic characteristic is stably maintained and inherited and then seeds harvested to ensure expression of the desired phenotypic

15 characteristic has been achieved.

The methods of the invention involve introducing a nucleotide construct into a plant. By "introducing" is intended presenting to the plant the nucleotide construct in such a manner that the construct gains access to the interior of a cell of the plant. The methods of the invention do not depend on a particular method for introducing a

20 nucleotide construct to a plant, only that the nucleotide construct gains access to the interior of at least one cell of the plant. It is recognized that certain embodiments of the invention do not depend on the stable incorporation of the *MSH2* nucleotide sequences of the invention into the genome of an organism.

Methods for introducing nucleotide constructs into plants are known in the art

25 including, but not limited to, stable transformation methods, transient transformation methods, and virus-mediated methods. By "stable transformation" is intended that the nucleotide construct introduced into a plant integrates into the genome of the plant and is capable of being inherited by progeny thereof. By "transient transformation" is intended that a nucleotide construct introduced into a plant does not integrate into the genome of

30 the plant.

The nucleotide constructs of the invention can be introduced into plants by contacting plants with a virus or viral nucleic acids. Generally, such methods involve incorporating a nucleotide construct of the invention within a viral DNA or RNA molecule. It is recognized that an MSH2 of the invention may be initially synthesized as
5   part of a viral polyprotein, which later may be processed by proteolysis *in vivo* or *in vitro* to produce the desired recombinant protein. Further, it is recognized that promoters of the invention also encompass promoters utilized for transcription by viral RNA polymerases. Methods for introducing nucleotide constructs into plants and expressing a protein encoded therein, involving viral DNA or RNA molecules, are known in the art.
10   See, for example, U.S. Patent Nos. 5,889,191, 5,889,190, 5,866,785, 5,589,367 and 5,316,931; herein incorporated by reference.

It is recognized that with these nucleotide sequences, antisense constructions, complementary to at least a portion of the messenger RNA (mRNA) for the *MSH2* sequences can be constructed. Antisense nucleotides are constructed to hybridize with
15   the corresponding mRNA. Modifications of the antisense sequences may be made as long as the sequences hybridize to and interfere with expression of the corresponding mRNA. In this manner, antisense constructions having 70%, preferably 80%, more preferably 85% sequence identity to the corresponding antisensed sequences may be used. Furthermore, portions of the antisense nucleotides may be used to disrupt the expression
20   of the target gene. Generally, sequences of at least 50 nucleotides, 100 nucleotides, 200 nucleotides, or greater may be used.

The nucleotide sequences of the present invention may also be used in the sense orientation to suppress the expression of endogenous genes in plants. Methods for suppressing gene expression in plants using nucleotide sequences in the sense orientation
25   are known in the art. The methods generally involve transforming plants with a DNA construct comprising a promoter that drives expression in a plant operably linked to at least a portion of a nucleotide sequence that corresponds to the transcript of the endogenous gene. Typically, such a nucleotide sequence has substantial sequence identity to the sequence of the transcript of the endogenous gene, preferably greater than
30   about 65% sequence identity, more preferably greater than about 85% sequence identity,

most preferably greater than about 95% sequence identity. See, U.S. Patent Nos. 5,283,184 and 5,034,323; herein incorporated by reference.

The use of the term "nucleotide constructs" herein is not intended to limit the present invention to nucleotide constructs comprising DNA. Those of ordinary skill in

5   the art will recognize that nucleotide constructs, particularly polynucleotides and oligonucleotides, comprised of ribonucleotides and combinations of ribonucleotides and deoxyribonucleotides may also be employed in the methods disclosed herein. Thus, the nucleotide constructs of the present invention encompass all nucleotide constructs that can be employed in the methods of the present invention for transforming plants

10   including, but not limited to, those comprised of deoxyribonucleotides, ribonucleotides, and combinations thereof. Such deoxyribonucleotides and ribonucleotides include both naturally occurring molecules and synthetic analogues. The nucleotide constructs of the invention also encompass all forms of nucleotide constructs including, but not limited to, single-stranded forms, double-stranded forms, hairpins, stem-and-loop structures, and the

15   like.

Furthermore, it is recognized that the methods of the invention may employ a nucleotide construct that is capable of directing, in a transformed plant, the expression of at least one protein, or at least one RNA, such as, for example, an antisense RNA that is complementary to at least a portion of an mRNA. Typically such a nucleotide construct

20   is comprised of a coding sequence for a protein or an RNA operably linked to 5' and 3' transcriptional regulatory regions. Alternatively, it is also recognized that the methods of the invention can employ a nucleotide construct that is not capable of directing, in a transformed plant, the expression of a protein or an RNA.

In addition, it is recognized that methods of the present invention do not depend

25   on the incorporation of the entire nucleotide construct into the genome, only that the plant or cell thereof is altered as a result of the introduction of the nucleotide construct into a cell. In one embodiment of the invention, the genome may be altered following the introduction of the nucleotide construct into a cell. For example, the nucleotide construct, or any part thereof, can incorporate into the genome of the plant. For the

30   present invention, alterations to the genome include, but are not limited to, additions,

deletions, and substitutions of nucleotides in the genome. While the methods of the present invention do not depend on additions, deletions, or substitutions of any particular number of nucleotides, it is recognized that such additions, deletions, or substitutions comprise at least one nucleotide.

5   The nucleotide constructs of the invention also encompass nucleotide constructs that may be employed in methods for altering or mutating a genomic nucleotide sequence in an organism, including, but not limited to, chimeraplasts, chimeric vectors, chimeric mutational vectors, chimeric repair vectors, mixed-duplex oligonucleotides, self-complementary chimeric oligonucleotides, and recombinogenic oligonucleobases. Such

10   nucleotide constructs and methods of use, such as, for example, chimeraplasty, are known in the art. Chimeraplasty involves the use of such nucleotide constructs to introduce site-specific changes into the sequence of genomic DNA within an organism. See, U.S. Patent Nos. 5,565,350; 5,731,181; 5,756,325; 5,760,012; 5,795,972; and 5,871,984; all of which are herein incorporated by reference. See also, WO 98/49350, WO 99/07865, WO

15   99/25821, and Beetham *et al.* (1999) *Proc. Natl. Acad. Sci. USA* 96:8774-8778; herein incorporated by reference.

The present invention may be used for transformation of any plant species, including, but not limited to, monocots and dicots. Examples of plant species of interest include, but are not limited to, corn (*Zea mays*), *Brassica* sp. (e.g., *B. napus, B. rapa, B.*

20   *juncea*), particularly those *Brassica* species useful as sources of seed oil, alfalfa (*Medicago sativa*), rice (*Oryza sativa*), rye (*Secale cereale*), sorghum (*Sorghum bicolor, Sorghum vulgare*), millet (e.g., pearl millet (*Pennisetum glaucum*), proso millet (*Panicum miliaceum*), foxtail millet (*Setaria italica*), finger millet (*Eleusine coracana*)), sunflower (*Helianthus annuus*), safflower (*Carthamus tinctorius*), wheat (*Triticum aestivum*), soybean (*Glycine*

25   *max*), tobacco (*Nicotiana tabacum*), potato (*Solanum tuberosum*), peanuts (*Arachis hypogaea*), cotton (*Gossypium barbadense, Gossypium hirsutum*), sweet potato (*Ipomoea batatus*), cassava (*Manihot esculenta*), coffee (*Coffea* spp.), coconut (*Cocos nucifera*), pineapple (*Ananas comosus*), citrus trees (*Citrus* spp.), cocoa (*Theobroma cacao*), tea (*Camellia sinensis*), banana (*Musa* spp.), avocado (*Persea americana*), fig (*Ficus casica*),

30   guava (*Psidium guajava*), mango (*Mangifera indica*), olive (*Olea europaea*), papaya

(*Carica papaya*), cashew (*Anacardium occidentale*), macadamia (*Macadamia integrifolia*), almond (*Prunus amygdalus*), sugar beets (*Beta vulgaris*), sugarcane (*Saccharum* spp.), oats, barley, vegetables, ornamentals, and conifers.

Vegetables include tomatoes (*Lycopersicon esculentum*), lettuce (e.g., *Lactuca*
5   *sativa*), green beans (*Phaseolus vulgaris*), lima beans (*Phaseolus limensis*), peas (*Lathyrus* spp.), and members of the genus *Cucumis* such as cucumber (*C. sativus*), cantaloupe (*C. cantalupensis*), and musk melon (*C. melo*). Ornamentals include azalea (*Rhododendron* spp.), hydrangea (*Macrophylla hydrangea*), hibiscus (*Hibiscus rosasanensis*), roses (*Rosa* spp.), tulips (*Tulipa* spp.), daffodils (*Narcissus* spp.), petunias (*Petunia hybrida*), carnation
10 (*Dianthus caryophyllus*), poinsettia (*Euphorbia pulcherrima*), and chrysanthemum.

Conifers that may be employed in practicing the present invention include, for example, pines such as loblolly pine (*Pinus taeda*), slash pine (*Pinus elliotii*), ponderosa pine (*Pinus ponderosa*), lodgepole pine (*Pinus contorta*), and Monterey pine (*Pinus radiata*); Douglas fir (*Pseudotsuga menziesii*); Western hemlock (*Tsuga canadensis*); Sitka
15 spruce (*Picea glauca*); redwood (*Sequoia sempervirens*); true firs such as silver fir (*Abies amabilis*) and balsam fir (*Abies balsamea*); and cedars such as Western red cedar (*Thuja plicata*) and Alaska yellow-cedar (*Chamaecyparis nootkatensis*).

Preferably, plants of the present invention are crop plants (for example, corn, alfalfa, sunflower, *Brassica*, soybean, cotton, safflower, peanut, sorghum, wheat, millet, tobacco,
20 etc.), more preferably corn and soybean plants, yet more preferably corn plants.

The *MSH2* promoter sequences of the invention can be used in methods for regulating gene expression in a plant. The methods of the invention involve operably linking an MSH2 promoter sequence of the invention to a second sequence wherein the MSH2 promoter allows the transcription of the second sequence. While the invention
25 does not depend on a particular second sequence, preferred sequences are coding sequences. Such preferred sequences can additionally comprise 3' and 5' untranslated regions associated with the coding sequence. Such preferred sequences can be operably linked in either the sense or antisense orientation depending on the desired outcome.

The *MSH2* promoter nucleotide sequences and methods disclosed herein are
30 useful in regulating expression of any heterologous nucleotide sequence in a host plant in

order to vary the phenotype of a plant. The methods of the invention involve operably linking an *MSH2* promoter nucleotide sequence of the invention to heterologous nucleotide sequence wherein the *MSH2* promoter allows the transcription of the heterologous nucleotide sequence.

5   Various changes in phenotype are of interest including modifying the fatty acid composition in a plant, altering the amino acid content of a plant, altering a plant's pathogen defense mechanism, and the like. These results can be achieved by providing expression of heterologous products or increased expression of endogenous products in plants. Alternatively, the results can be achieved by providing for a reduction of

10   expression of one or more endogenous products, particularly enzymes or cofactors in the plant. These changes result in a change in phenotype of the transformed plant.

        Genes of interest are reflective of the commercial markets and interests of those involved in the development of the crop. Crops and markets of interest change, and as developing nations open up world markets, new crops and technologies will emerge also.

15   In addition, as our understanding of agronomic traits and characteristics such as yield and heterosis increase, the choice of genes for transformation will change accordingly. General categories of genes of interest include, for example, those genes involved in information, such as zinc fingers, those involved in communication, such as kinases, and those involved in housekeeping, such as heat shock proteins. More specific categories of

20   transgenes, for example, include genes encoding important traits for agronomics, insect resistance, disease resistance, herbicide resistance, sterility, grain characteristics, and commercial products. Genes of interest include, generally, those involved in oil, starch, carbohydrate, or nutrient metabolism as well as those affecting kernel size, sucrose loading, and the like.

25   Agronomically important traits such as oil, starch, and protein content can be genetically altered in addition to using traditional breeding methods. Modifications include increasing content of oleic acid, saturated and unsaturated oils, increasing levels of lysine and sulfur, providing essential amino acids, and also modification of starch. Hordothionin protein modifications are described in U.S. Application Serial No.

30   08/838,763, filed April 10, 1997; and U.S. Patent Nos. 5,703,049, 5,885,801, and

5,885,802, herein incorporated by reference. Another example is lysine and/or sulfur rich seed protein encoded by the soybean 2S albumin described in U.S. Patent No. 5,850,016, and the chymotrypsin inhibitor from barley, described in Williamson *et al.* (1987) *Eur. J. Biochem. 165*:99-106, the disclosures of which are herein incorporated by reference.

5   Derivatives of the coding sequences can be made by site-directed mutagenesis to increase the level of preselected amino acids in the encoded polypeptide. For example, the gene encoding the barley high lysine polypeptide (BHL) is derived from barley chymotrypsin inhibitor, U.S. Application Serial No. 08/740,682, filed November 1, 1996, and WO 98/20133, the disclosures of which are herein incorporated by reference. Other

10   proteins include methionine-rich plant proteins such as from sunflower seed (Lilley *et al.* (1989) *Proceedings of the World Congress on Vegetable Protein Utilization in Human Foods and Animal Feedstuffs*, ed. Applewhite (American Oil Chemists Society, Champaign, Illinois), pp. 497-502; herein incorporated by reference); corn (Pedersen *et al.* (1986) *J. Biol. Chem. 261*:6279; Kirihara *et al.* (1988) *Gene 71*:359; both of which are

15   herein incorporated by reference); and rice (Musumura *et al.* (1989) *Plant Mol. Biol. 12*:123, herein incorporated by reference). Other agronomically important genes encode latex, Floury 2, growth factors, seed storage factors, and transcription factors.

        Insect resistance genes may encode resistance to pests that have great yield drag such as rootworm, cutworm, European Corn Borer, and the like. Such genes include, for

20   example, *Bacillus thuringiensis* toxic protein genes (U.S. Patent Nos. 5,366,892; 5,747,450; 5,736,514; 5,723,756; 5,593,881; and Geiser *et al.* (1986) *Gene 48*:109); lectins (Van Damme *et al.* (1994) *Plant Mol. Biol. 24*:825); and the like.

        Genes encoding disease resistance traits include detoxification genes, such as against fumonosin (U.S. Patent No. 5,792,931); avirulence (avr) and disease resistance

25   (R) genes (Jones *et al.* (1994) *Science 266*:789; Martin *et al.* (1993) *Science 262*:1432; and Mindrinos *et al.* (1994) *Cell 78*:1089); and the like.

        Herbicide resistance traits may include genes coding for resistance to herbicides that act to inhibit the action of acetolactate synthase (ALS), in particular the sulfonylurea-type herbicides (e.g., the acetolactate synthase (ALS) gene containing mutations leading

30   to such resistance, in particular the S4 and/or Hra mutations), genes coding for resistance

to herbicides that act to inhibit action of glutamine synthase, such as phosphinothricin or basta (e.g., the bar gene), or other such genes known in the art. The *bar* gene encodes resistance to the herbicide basta, the *nptII* gene encodes resistance to the antibiotics kanamycin and geneticin, and the ALS-gene mutants encode resistance to the herbicide

5   chlorsulfuron.

Sterility genes can also be encoded in an expression cassette and provide an alternative to physical detasseling. Examples of genes used in such ways include male tissue-preferred genes and genes with male sterility phenotypes such as QM, described in U.S. Patent No. 5,583,210. Other genes include kinases and those encoding compounds

10   toxic to either male or female gametophytic development.

The quality of grain is reflected in traits such as levels and types of oils, saturated and unsaturated, quality and quantity of essential amino acids, and levels of cellulose. Modified hordothionin proteins are described in U.S. Patent Nos.: 5, 990,389; 5,703,049; 5,885,801; and 5,885,802.

15   Commercial traits can also be encoded on a gene or genes that could increase for example, starch for ethanol production, or provide expression of proteins. Another important commercial use of transformed plants is the production of polymers and bioplastics such as described in U.S. Patent No. 5,602,321. Genes such as β-Ketothiolase, PHBase (polyhydroxyburyrate synthase), and acetoacetyl-CoA reductase

20   (see Schubert *et al.* (1988) *J. Bacteriol. 170*:5837-5847) facilitate expression of polyhyroxyalkanoates (PHAs).

Exogenous products include plant enzymes and products as well as those from other sources including prokaryotes and other eukaryotes. Such products include enzymes, cofactors, hormones, and the like. The level of proteins, particularly modified

25   proteins having improved amino acid distribution to improve the nutrient value of the plant, can be increased. This is achieved by the expression of such proteins having enhanced amino acid content.

The invention further provides host cells transformed with at least one of the *MSH2* nucleotide sequences of the invention. The host cells of the invention can be from

30   any organism including, but not limited to, bacteria, fungi, animals and plants. A

nucleotide construct comprising an MSH2 nucleotide sequence of the invention can be introduced into a host cell by any transformation methods known in the art. Such an introduced nucleotide construct can be stably integrated in the genome of the host cell or be present within the host cell in non-integrated form such as, for example, a plasmid, a

5   cosmid, an artificial chromosome, or other vector. Expression cassettes can be constructed which include the nucleotide constructs of interest operably linked with the transcriptional and translational regulatory signals for expression of the nucleotide construct within the desired host cell.

Transcriptional and translational regulatory signals include, but are not limited to,

10   promoters, transcriptional initiation start sites, operators, activators, enhancers, other regulatory elements, ribosomal binding sites, an initiation codon, termination signals, and the like. See, for example, U.S. Patent 5,039,523; U.S. Patent No. 4,853,331; EPO 0480762A2; Sambrook *et al. supra*; Molecular Cloning, a Laboratory Manual, Maniatis *et al.* (eds) Cold Spring Harbor Laboratory, Cold Spring Harbor, NY (1982); Advanced

15   Bacterial Genetics, Davis *et al.* (eds.) Cold Spring Harbor Laboratory, Cold Spring Harbor, NY (1980); and the references cited therein.

The host cells of the invention can be used as a source of MSH2 proteins for the isolation or purification of such proteins. If desired and using methods known to those of ordinary skill in the art, expression systems can be designed in such a manner to cause

20   the MSH2 proteins to be secreted outside the cytoplasm of a bacterium, such as, for example, the gram-negative bacterium, *E. coli*. Advantages of having MSH2 secreted include, but are not limited to, (1) a reduction in, or avoidance of, potential cytotoxic effects of MSH2, and (2) an improvement in the efficiency of purification of MSH2. By "improvement in the efficiency of purification" is intended an improvement in at least

25   one aspect of protein purification, including by not limited to, decreased cost of purification of a unit amount of protein, increased recovery of protein per purification attempt, increased recovery of active protein per purification attempt, and increased protein yield per bacterial cell or volume of culture broth. In addition, the invention encompasses fusion proteins comprising an MSH2 of the invention and a epitope or tag

30   that can be used to facilitate purification and/or detection of such a fusion protein. Such

epitopes or tags, and methods of use, are known in the art and include, for example, the polyhistidine-tag (his-tag).

MSH2 can be modified for secretion in *E. coli* by, for example, fusing an appropriate *E. coli* signal peptide to the amino-terminal end of the MSH2 protein. Signal

5   peptides recognized by *E. coli* can be found in proteins already known to be secreted in *E. coli*, such as, for example the OmpA protein (J. Ghrayeb *et al.* (1984) *EMBO J.*, *3*:2437-2442). OmpA is a major protein of the *E. coli* outer membrane and thus its signal peptide is thought to be efficient in the translocation process. Also, the OmpA signal peptide does not need to be modified before processing as may be necessary for other

10   signal peptides, such as, for example the lipoprotein signal peptide (G. Duffaud *et al.* (1987) *Methods in Enzymology 153*:492).

The following examples are presented by way of illustration, not by way of limitation.

15

EXPERIMENTAL

EXAMPLE 1: Isolation of an *MSH2* cDNA from Tobacco

20   <u>Materials and Methods</u>

RNA extraction and RT-PCR. Total RNA was isolated by the LiCl method. Briefly, the method involves extraction of a tissue sample in 0.2 M NaOAC pH 5.2, 1% SDS, 0.5 mM EDTA pH 8.0. The resulting tissue slurry is extracted sequentially in phenol,

25   phenol:chloroform (50:50), and chloroform. The resulting aqueous phase is precipitated in 2.5 M LiCl overnight at 4°C. Two to five µg of total RNA was reverse transcribed at 42°C using 200 units MMLV reverse transcriptase (Promega, Madison, WI) in the buffer supplied, supplemented with 10 mM DTT and 1 mM dNTPs, in a 25 µL total volume.

Degenerate RT-PCR.   Degenerate oligonucleotides were designed against highly

30   conserved MutS family signature amino acid motifs; TGPNM (SEQ ID NO:22) and

FATHY (SEQ ID NO:23) (Reenan and Kolodner (1992) *Genetics 132*:963-973). A typical PCR utilized 5 μL of a cDNA synthesis reaction (described above). PCR conditions consisted of 30-35 cycles of 96°C for 13 sec., 48°C for 45 sec. and 72°C for 30 sec. Aliquots of the PCR products were ligated into pGemT-Easy (Promega) without

5   purification. Putative tobacco *MSH2* subclones were sequenced or tested for hybridization to heterologous DNA probes. Several identical tobacco *MSH2* clones were identified by cross hybridization to an *Arabidopsis thaliana* var. Columbia genomic PCR product (Culligan and Hays (1997) *Plant Physiol. 115*:833-839) (Accession No. AF003005). The heterologous *Arabidopsis* probe was amplified from *Arabidopsis* genomic DNA using

10   two oligonucleotides designated as AT-TG (5' GTAACAGGGCCTAACATGGG 3') (SEQ ID NO:24) and AT-FATH (5' GGAAGTGAGTAGCAAACAG 3')(SEQ ID NO:25). This resulting probe contains sequences encoding the *Msh* family signature region, as well as three introns (bases X-Y). Cloned PCR products were sequenced using Sequenase 2.0 (Amersham), or on an ABI 377 automated sequencer.

15

3' RACE

Five μg total RNA was reverse transcribed according to the Ready-2-go cDNA synthesis kit (Pharmacia). The PCR employed a gene-specific oligo "TG internal" (5' CAGGCCCTAACATGGGTGG 3') (SEQ ID NO:26), in conjunction with the modified

20   oligo dT included in the kit. Eleven μL of the 33 μL cDNA synthesis reaction was used for PCR according the manufacturer's instructions, except that the PCR was supplemented with 1 μL 5 mM dNTPs. Typical PCR conditions consisted of 35 cycles of 96°C for 13 sec., 55°C for 40 sec., 72°C for 1.5 min. Aliquots of the PCR products were ligated into pGemT-Easy without purification. Plasmid clones were sequenced on an ABI 377 automated

25   sequencer.

Generation of a full-length tobacco MSH2 cDNA. The tobacco *MSH2* sequence was completed using a combination of 5' RACE, RT-PCR and inverse PCR (IPCR). Several upstream oligonucleotides were designed against amino acid motifs common to the *Arabidopsis thaliana* and *Saccharomyces cerevisiae* MSH2 proteins. These motifs included

30   Y, MWLQP, E. The sequences of upstream oligonucleotides against these putatively

conserved amino acid motifs were identical to the known *A. thaliana* cDNA sequence (Culligan and Hays (1997) *Plant Physiol. 115*:833-839). In each RT-PCR, an oligonucleotide designed to hybridize to the known tobacco *MSH2* sequence was used for the reverse transcription, and a nested oligonucleotide was used in conjunction with an

5 upstream oligonucleotide for PCR. 5' RACE conditions followed the strategy of the Cap Finder 5' RACE kit (BRL). In short, purified reverse transcribed cDNA was 3' dC-tailed with terminal transferase (Promega) and dCTP. PCR of dC-tailed cDNA employed gene specific oligonucleotides and the G-anchor oligonucleotide (seq), using a cycling profile of 96°C for 13 sec., 55-60°C for 40 sec., 72° for 45 sec. IPCR was conducted on tobacco Nt-1

10 cell DNA digested with *Xba* I and recircularized by ligation, as reported elsewhere (Ochman *et al.* (19880 *Genetics 149*:641-650). Recircularized genomic DNA was amplified with two oligonucleotides; IPCR3 (5' AATGAAATGCAAGATTCTCC 3') (SEQ ID NO:27)and IPCR4 (5' GAAGCTTGCTCTGTTCCTCC 3') (SEQ ID NO:28). PCR products were cloned by ligation into pGemT-Easy. Plasmid clones were sequenced on an ABI 310

15 automated sequencer, using the Big Dye Terminator kit (Perkin-Elmer, CT).

 DNA blot hybridizations. Plasmid clones (or PCR products) were transferred from agarose gels to Nytran Plus membranes using a vacuum blotter. The DNA was transferred from the gel for 1 hour in 0.4 M NaOH, and UV crosslinked to the membrane. Plasmid blots were hybridized for 2-5 hours at 65°C, using ^{32}P-labelled, random-primed probes

20 (Amersham) and washed twice in 40 mM NaPi (pH 7.2), 1 mM EDTA (pH 8) and 1% SDS. Radioactive signals were detected using a PhosphorImager (Molecular Dynamics, Sunnyvale, CA).

 Genomic DNA was isolated from Nt-1 cells by the CTAB method and treated with pronase (Sambrook *et al.* (1989) Molecular Cloning: A Laboratory Manual, 2d ed., Cold

25 Spring Harbor Laboratory Press, Plainview, New York). Approximately 15 µg of tobacco DNA was restriction digested overnight at 37°C in a 600 µL volume. Digestion products were precipitated with isopropanol and electrophoresed on a 0.7% agarose gel for 12 hrs. The gel was transferred to a Nytran Plus membrane by capillary blot in 12X SSC (Sambrook *et al.* (1989) Molecular Cloning: A Laboratory Manual, 2d ed., Cold Spring

30 Harbor Laboratory Press, Plainview, New York). The UV crosslinked membranes were

hybridized to ^{32}P-labelled (NEN-DuPont) random primed probes at >10[9] counts/mL, in a hybridization volume of 1-2 mL, for 14-18 hours. Radioactive signals were detected using a PhosphorImager or by exposure to film at −80°C with an intensifying screen for 3-5 days.

5   Results

Degenerate oligonucleotides were designed against two highly conserved MutS family signature amino acid motifs; TGPNM (5' ACNGGNCCNAAYATGGG 3') (SEQ ID NO:29) and FATHY (5' TGYAARTGNGTNCGRAA 3') (SEQ ID NO:30) (Reenan and Kolodner (1992) *Genetics 132*:963-973). Degenerate RT-PCR product subclones were

10   screened by hybridization to an *A. thaliana* probe, comprising the corresponding region of the *A. thaliana MSH2* gene. Three candidate subclones were identified by southern analysis. The amplified sequence was highly homologous to the *A. thaliana MSH2*, and more similar to *MSH2s* than other *Msh* genes. A gene-specific oligonucleotide against the ATP binding site (5' CAGGCCCTAACATGGGTGG 3') (SEQ ID NO:31) was designed

15   for 3' RACE. A 1.032 kb 3' RACE product was identified by hybridization to the previously identified sequences, which comprised the 3' end of the *MSH2* coding region, as well as the 3' UTR. Additional 5' *MSH2* cDNA sequences were obtained in a manner analogous to a chromosome walk utilizing degenerate RT-PCR and 5' RACE.

Two 5' degenerate oligonuclotides were designed to correspond to nucleotide

20   sequences encoding the amino acid motifs DYYTAH (SEQ ID NO:32)
(5' GATTATTATACAGCTCATGG 3') (SEQ ID NO:33) and MWLKQP (SEQ ID NO:34) (5'□ ATGTGGCTGAAACAACC 3') (SEQ ID NO:35), which are shared among the human, yeast and Arabidopsis MSH2 proteins. Whenever practical, an oligonucleotide designed to hybridize to the known tobacco *MSH2* sequence was used for the reverse

25   transcription and a nested oligonucleotide was used in conjunction with an upstream oligonucleotide for PCR. A typical PCR utilized 5 mL of a cDNA synthesis reaction, and followed the reaction condition parameters described above. The 3' oligonucleotides ultilized for degenerate RT-PCR of the "M" product (to be used in conjunction with SEQ ID NO:34) were designated as the "unique R1" primer

30   (5' CTTATGTCCATTGTCTCCATTC 3' (SEQ ID NO:36) for cDNA synthesis) and the

"unique R2" nested primer (5' GTCCATTGTCTCCATTCTTG 3', (SEQ ID NO:37) for

PCR of cDNA). The 3' oligonucleotides for degenerate RT-PCR of the "D" product (used

in conjunction with the 5' DYYTAH oligonucleotide (SEQ ID NO:33) were designated as

the "22a" primer (5' GCACCCCAAAGCGCCTGATG 3' (SEQ ID NO:38), for cDNA

5   synthesis) and the "22b" nested primer (5' CTGATGCACATTCGAACCCAGAG 3' (SEQ

ID NO:39), for PCR of cDNA). PCR conditions were 96°C for 17 s, 48-55°C for 45 s,

72°C for 30 s to 2 min for each cycle, and with a total of 30-35 cycles conducted using a

thermocycler (ABI 9700). The degenerate RT-PCR products (and/or their subclones) were

identified by hybridization to previously characterized, overlapping *NtMSH2* sequences.

10   Sequence analysis of subcloned 5' RACE products generated from the 5' ends of the

transcripts revealed several nucleotide polymorphisms within the coding region as well as

the 5' untranslated regions (UTRs) of the *MSH2* transcripts. Among the 5' UTR

polymorphisms between the alleles is a homonucleotide run of either nine or eleven

consecutive adenines. In each 5' UTR, the last base of the homoA run is the A of the start

15   codon (ATG), leading to an atypical translation initiation context for the *MSH2* mRNAs.

        To further characterize nucleotide polymorphisms found in the 5' ends of the

MSH2 transcripts, a 169 bp section of the *MSH2* was amplified by RT-PCR and the

products were subcloned and sequenced. The DNA sequence of these RT-PCR products

fell into distinct groups based upon nucleotide polymorphisms at six sites (Figure 1).

20   This analysis revealed two different MSH2 proteins as the nucleotide polymorphism at

cDNA position 145, T or G, leads to non-degenerate codons; TCT, encoding serine, or

GCT, encoding alanine at amino acid position 49. Additionally, two polymorphic

(degenerate) versions of the sequence containing the alanine codon were identified. The

presence of two distinct protein coding regions and multiple polymorphic mRNAs was

25   confirmed by sequencing PCR products amplified from tobacco genomic DNA as

described below.

        Approximately 15 µg of tobacco genomic DNA, derived from Nt-1 cells, was

digested with the restriction enzymes Hind III, Sst I or Xba I and blotted to a nylon

membrane using standard molecular biology methods (Sambrook *et al.* (1989) Molecular

30   Cloning: A Laboratory Manual, 2d ed., Cold Spring Harbor Laboratory Press, Plainview,

New York). The hybridization pattern of the 5' *MSH2* cDNA probe indicated the presence of two similar *MSH2* loci (data not shown). The probe hybridized to two species in lane 1 (*Hind* III) and two similar sized species in lane 3 (Xba I). A 3' *MSH2* cDNA probe (panel b), which contains the *Msh* family signature sequences, hybridizes to a large number of

5 bands, suggesting hybridization to other members of an as yet unidentified *Msh* gene family.

EXAMPLE 2: Identification of *MSH2* Regulatory Elements and
Demonstration of *MSH2* Promoter Activity

10 By comparing the restriction map generated from the tobacco *MSH2* cDNA sequence and the data from the genomic Southern blots, it was expected that the approximately 1.7 kb *XbaI* fragments identified by the Southern analysis (Example 1) should contain a substantial portion of the 5' end of the genes. Inverse PCR (IPCR) was utilized to amplify flanking *MSH2* sequences (SEQ ID NOS:5-12). Oligonucleotides were

15 designed to hybridize just 3' of the start codon and approximately 100 bases 5' of the first *XbaI* site identified in the cDNA sequence. IPCR amplified two distinct promoter-like sequences, designated class I and class II, as well as the adjacent 5' UTR sequences. The sequences of these regulatory elements are polymorphic at several sites, summarized in (SEQ ID NOS:5-12). Notably, the class I promoter element contains a canonical CCAAT

20 box; the corresponding sequence of the class II element is CCAAC. Additionally, the class I *MSH2* promoter contains a ten base insertion that is completely absent in the class II element. This ten base insertion is nearly a perfect direct repeat, consistent with a role in DNA:protein interaction. Each class of *MSH2* promoter has an identical TATA element. Nucleotide polymorphisms previously identified within the distinct 5' UTRs were

25 confirmed, and two additional SNPs (single nucleotide polymorphisms) were found in sequences between the TATA element and the start codon. These could not be definitively ascribed to the promoter or the coding region, as efforts to conduct primer extension were unsuccessful.

Each *MSH2* regulatory element was fused to a *uidA* reporter and bombarded into

30 lawns of Nt-1 cells. Bombarded cells were assayed for accumulation of GUS protein by

histochemical assay and for the transcription of *MSH2:uidA* mRNA by RT-PCR (data not shown). GUS staining in the bombarded cells and RT-PCR analysis demonstrated that each promoter element is competent to transcribe mRNA (data not shown).

5   EXAMPLE 3: Amplification and Sequencing of Genomic DNAs

Corresponding to the 5' Ends of the *MSH2* Alleles

To establish which promoter sequences drive the polymorphic mRNAs, oligonucleotides were designed to amplify regions between the promoters and nucleotide

10   179 of the cDNA sequence. Genomic PCR products were ligated into pGem-T Easy (Promega Corp., Madison, WI), and the resulting plasmid subclones were sequenced. Genomic PCR products representing the two promoter classes as well as each of the three classes of polymorphic mRNA (SEQ ID NOS:13-15) were identified through DNA sequencing. Each product also contained three introns, which are present at the same

15   location in the cDNA sequence with respect to the start codon, but exhibit varying levels of polymorphism when compared to one another. When the promoters (SEQ ID NOS:5-12), 5' UTRs (SEQ ID NOS:13-15), coding regions (SEQ ID NOS:1 and 3) and introns (SEQ ID NOS:16-21) were compared, it was determined that four distinct *MSH2* genes had been identified. Three of the genes have a class II promoter, while only one gene bearing a class I

20   promoter was identified. The transcript linked to the class I promoter encodes serine at amino acid 49, and has the G/C rich set of SNPs. Three distinct genes with class II promoters were identified. One gene contains the G/C rich set of SNPs, and encodes serine at amino acid 49. The other two genes have the A/T rich set of SNPs, but they differ by a two nucleotide base polymorphism resulting in either an alanine or serine codon at position

25   49. In addition to the two base polymorphism, there are substantial differences between the introns of these two genes with the A/T rich SNPs set. For example, the second intron of the gene encoding alanine (107 nucleotides in length) and the second intron of the gene encoding serine (105 nucleotides) differ by two insertion/deletions and eight SNPs.

EXAMPLE 4: *MSH2* Expression Levels and Distribution

<u>Materials and Methods</u>

    Antibody production and immunoblotting. The $(His)_6$ vector pQE31 (Qiagen;

5   Valencia, CA) was restricted with BamHI and filled with the Klenow large fragment

(Sambrook *et al*. (1989) Molecular Cloning: A Laboratory Manual, 2d ed., Cold Spring

Harbor Laboratory Press, Plainview, New York) to create a blunt end, then digested with

Hind III (NEB). A restriction fragment of the tobacco cDNA sequence, spanning the

codons for amino acids 670-939 was ligated into the prepared vector as a Klenow filled *Sal*

10   I/*Hind* III fragment to generate the $(His)_6C$ fusion protein.

    The $(His)_6C$ fusion protein was purified using the NTA-nickel matrix under

denaturing conditions following the manufacturer's recommendations (Qiagen, Valencia,

CA). The purified protein was dialyzed against PBS (phosphate-buffered saline), in the

presence of 10 mM 2-mercaptoethanol and 0.3 mM PMSF. The dialyzed protein was

15   concentrated threefold using a Centricon spin concentrator, according to the manufacturers

instructions (Amicon). Rabbit antisera was produced by the Cornell Veterinary School

using the purified protein.

    Total proteins were extracted from frozen pulverized tobacco tissues in 20 mM Na

phosphate (pH 6.6), 100 mM NaCl, 1 mM EDTA pH 8, 0.1 mM β-mercaptoethanol, 0.5

20   mM PMSF and 15 mg/L leupeptin. Thirty-five µg samples of protein isolated separately

from tobacco Nt-1 cells and from tobacco leaf, root, and flower tissues were electrophoresed

through 8% polyacrylamide gels and transferred to Transblot nitrocellulose membranes

(BioRad) using a Transblot apparatus (BioRad), according to the manufacturer's

instructions. Rabbit anti-tMSH2 immune serum was incubated with membranes at a 1-900

25   dilution for 3 hours at 25°C in TBS (Tris-buffered saline) (Sambrook *et al*. (1989)

Molecular Cloning: A Laboratory Manual, 2d ed., Cold Spring Harbor Laboratory Press,

Plainview, New York) with 5% dry milk and 0.05% NaN_3. The primary antibody was

detected using an anti-rabbit HRP-conjugated secondary antibody (Sigma).

    Relative RT-PCR. Three independently isolated five µg samples of total RNA were

30   prepared from flower, leaf and root tissues and then reverse transcribed at 42°C as described

above following RQ-DNAse treatment (Promega Corp., Madison, WI). A linear range of amplification was determined for each tissue (data not shown). Individual PCR reactions were conducted using 1 μL of each of the 25 μL cDNA syntheses, for 22, 24, 26 and 28 cycles. The cDNA synthesis employed the 3' RtB primer

5   (5' ACATATAGTTCAAGAGTACGGT 3') (SEQ ID NO:40). The resulting cDNA products were re-amplified with the 3' RtA primer
(3' GCTATTGTTTCAAACATGTTTC 3') (SEQ ID NO:41) and the 5'MSH primer
(5' TTGGAGGAACAGAGCAAGCTTC 3') (SEQ ID NO:42). Amplification products were electrophoresed and quantitated by measuring ethidium bromide fluorescence (Eagle

10   Eye, Stratagene). Ethidium bromide band intensities were plotted vs. cycle number for each tissue type, and linear ranges were established. To compare the samples from each tissue, a 1 μL volume of each cDNA synthesis was PCR amplified for 25 cycles.

Results

15   The levels of MSH2 protein were assessed in mature leaf, callus derived from leaf, root, flower (total), anther/pollen, stigma/style, ova petal, sepal and Nt-1 cells. Total protein was measured by the method of Bradford. Results from the immunoblotting of approximately 35 μg total protein samples indicated the presence of *MSH2* in all tobacco tissues tested. As a control to demonstrate the integrity of the protein samples, the

20   membranes were also incubated with antisera against ascorbate reductase. The accumulation of MSH2 varies substantially between tissues, and consistently accumulates to relatively higher levels in actively dividing tissues (data not shown). Several interesting trends were observed. The MSH2 protein seems to accumulate to higher levels in female gametophytic tissues compared to male gametophytic tissues (data not shown). Finally, Nt-1 cells, the

25   most actively dividing tissue of those tested, had a large accumulation of MSH2 protein. These data suggest that MSH2 protein accumulates in actively dividing cells, but is still present in non-dividing cells.

The accumulation of *MSH2* mRNA was measured utilizing the Invader Assay Invader Assay (Third Wave Technologies). (Lyamichev *et al.* (1998) *Nature Biotech.*

30   *17*:292-296).

EXAMPLE 5: Expression of the MSH2 N-terminal Region

Confers a Mutator Phenotype

5   <u>Materials and Methods</u>

Dominant negative overexpression of a tobacco MSH2 sequence in E. coli. The

(His)$_6$ vector pQE30 (Qiagen; Valencia, CA) was restricted with *Hind* III and filled with the

Klenow large fragment (Sambrook *et al.* (1989) Molecular Cloning: A Laboratory Manual,

2d ed., Cold Spring Harbor Laboratory Press, Plainview, New York) to create a blunt end,

10   then digested with *Bam*H I. An RT-PCR product of the *tMSH2* sequence was created using

the 22-base oligonucleotide and an oligonucleotide overlapping the *tMSH2* start codon

which generated a unique *Bam*H I restriction enzyme site just upstream of the ATG. This

RT-PCR product was ligated into pGem-T Easy. The resulting plasmid was digested with

*Eco*R I, blunted with Klenow, and subsequently *Bam*H I digested. This blunt-*Eco*R I

15   fragment encoding the first 265 amino acids of the tobacco MSH2 protein was ligated into

the *Bam*H I/blunt pQE30 described above to create (His)$_6$N. Fragments encoding the (His)$_6$-

MSH2 fusions ((His)$_6$C and (His)$_6$N) were sequenced on an ABI 310 automated sequencer.

E. coli cells (XL-1 blue, Stratagene) harboring pQE-MSH2 fusions or pKSII

(Stratagene) were grown for 6 hours in LB media containing 100 µg/mL ampicillin and 0.05

20   mM IPTG to induce production of the protein fusions or the α-peptide of β-galactosidase.

Cell culture dilutions were plated onto LB plates containing 100 µg/mL ampicillin to

determine cell viability. Mutated cells were selected on plates containing 100 µg/mL

ampicillin and 150µg/mL rifampicin (Reenan and Kolodner (1992) *Genetics 132:*963-973).

25   <u>Results</u>

The overexpression of a *MutS* homolog in an heterologous system can lead to a

dominant mutator phenotype. To determine if the tobacco MSH2 could cause a mutator

phenotype in *E. coli*, an (His)$_6$-tagged, IPTG-inducible plasmid clone (Qiagen, Valencia,

CA) was constructed by cloning the nucleotides 1-797 of SEQ ID NO: 1, which encodes the

30   N-terminal 265 amino acids of the tobacco MSH2 ((His)$_6$N) into pQE30 (Qiagen, Valencia,

CA). The N-terminus of MutS has been shown to interact directly with DNA (Malkov *et al.* (1997) *J. Biol. Chem.* 272: 23811-23817) via a DYYT motif. This amino acid motif is present in all identified MSH2 proteins, including the tobacco homologue. Parallel fluctuation analysis was performed on 11 independent XL-1 blue (Stratagene, La Jolla CA)

5   cultures containing either pBluescriptKS or (His)$_6$N plasmids. Mutation rates were determined by plating cells on LB plates containing 150 mg/L rifampicin and 100 mg/L ampicillin after 6 hrs. growth in 5 μM IPTG. The total number of viable cells in each culture was estimated by counting ampicillin-resistant colonies from dilutions of the cell cultures.

10

EXAMPLE 6: The Tobacco MSH2 Exists in

Higher Molecular Weight Complexes

Nuclear extracts derived from tobacco NT-1 cells were subjected to density

15   centrifugation in sucrose gradients. Seventeen fractions from 4 –19% sucrose gradients were collected, and gradient fractions containing MSH2 were identified by SDS-PAGE followed by immunoblotting. Parallel sucrose gradients containing marker proteins of known molecular weight (E. coli alkaline phosphatase (80 kDa), α-amylase (220 kDa), and apoferitin (450 kDa)) were used to approximate the molecular weight range of fractions

20   containing MSH2. Immunoblotting of gradient fractions revealed that MSH2 could be detected in disparate regions of the gradients, in fractions 3 and 4 (approximately 80 kDa) and also in fraction 9 (approximately 260 kDa).

EXAMPLE 7: Transformation and Regeneration of Transgenic Maize Plants

25

Immature maize embryos from greenhouse donor plants are bombarded with a plasmid containing an *MSH2* operably linked to a maize ubiquitin plus a plasmid containing the selectable marker gene PAT (Wohlleben *et al.* (1988) *Gene* 70:25-37) that confers resistance to the herbicide Bialaphos. Transformation is performed as follows.

30   Media recipes follow below.

Preparation of Target Tissue

The ears are surface sterilized in 30% Chlorox bleach plus 0.5% Micro detergent for 20 minutes, and rinsed two times with sterile water. The immature embryos are excised and placed embryo axis side down (scutellum side up), 25 embryos per plate, on

5   560Y medium for 4 hours and then aligned within the 2.5-cm target zone in preparation for bombardment.

Bombardment and Culture Media

Bombardment medium (560Y) comprises 4.0 g/l N6 basal salts (SIGMA C-1416), 1.0 ml/L Eriksson's Vitamin Mix (1000X SIGMA-1511), 0.5 mg/L thiamine HCl, 120.0

10   g/L sucrose, 1.0 mg/L 2,4-D, and 2.88 g/L L-proline (brought to volume with D-I H_20 following adjustment to pH 5.8 with KOH); 2.0 g/L Gelrite (added after bringing to volume with D-I H_20); and 8.5 mg/L silver nitrate (added after sterilizing the medium and cooling to room temperature). Selection medium (560R) comprises 4.0 g/L N6 basal salts (SIGMA C-1416), 1.0 ml/L Eriksson's Vitamin Mix (1000X SIGMA-1511), 0.5 mg/L

15   thiamine HCl, 30.0 g/L sucrose, and 2.0 mg/L 2,4-D (brought to volume with D-I H_20 following adjustment to pH 5.8 with KOH); 3.0 g/L Gelrite (added after bringing to volume with D-I H_20); and 0.85 mg/L silver nitrate and 3.0 mg/L bialaphos(both added after sterilizing the medium and cooling to room temperature).

Plant regeneration medium (288J) comprises 4.3 g/L MS salts (GIBCO 11117-

20   074), 5.0 ml/L MS vitamins stock solution (0.100 g nicotinic acid, 0.02 g/L thiamine HCl, 0.10 g/l pyridoxine HCL, and 0.40 g/L glycine brought to volume with distilled D-I H_2O) (Murashige and Skoog (1962) *Physiol. Plant. 15*:473), 100 mg/l myo-inositol, 0.5 mg/L zeatin, 60 g/L sucrose, and 1.0 ml/L of 0.1 mM abscisic acid (brought to volume with polished D-I H_2O after adjusting to pH 5.6); 3.0 g/L Gelrite (added after bringing to

25   volume with D-I H_2O); and 1.0 mg/L indoleacetic acid and 3.0 mg/L bialaphos (added after sterilizing the medium and cooling to 60°C). Hormone-free medium (272V) comprises 4.3 g/L MS salts (GIBCO 11117-074), 5.0 ml/L MS vitamins stock solution (0.100 g/L nicotinic acid, 0.02 g/L thiamine HCL, 0.10 g/L pyridoxine HCl, and 0.40 g/L glycine brought to volume with polished D-I H_2O), 0.1 g/L myo-inositol, and 40.0 g/L

30   sucrose (brought to volume with polished D-I H_2O after adjusting pH to 5.6); and 6 g/L

bacto-agar (added after bringing to volume with polished D-I H_2O), sterilized and cooled to 60° C.

Preparation of DNA

5 A plasmid vector comprising the *MSH2* operably linked to a maize ubiquitin is made. This plasmid DNA plus plasmid DNA containing a PAT selectable marker is precipitated onto 1.1 µm (average diameter) tungsten pellets using a $CaCl_2$ precipitation procedure as follows:

> 100 µL prepared tungsten particles in water
>
> 10 µL (1 µg) DNA in TrisEDTA buffer (1 µg total)
>
10 > 100 µL 2.5 M $CaCl_2$
>
> 10 µL 0.1 M spermidine

Each reagent is added sequentially to the tungsten particle suspension, while maintained on the multitube vortexer. The final mixture is sonicated briefly and allowed to incubate under constant vortexing for 10 minutes. After the precipitation period, the 15 tubes are centrifuged briefly, liquid removed, washed with 500 mL 100% ethanol, and centrifuged for 30 seconds. Again the liquid is removed, and 105 µL 100% ethanol is added to the final tungsten particle pellet. For particle gun bombardment, the tungsten/DNA particles are briefly sonicated and 10 µL spotted onto the center of each macrocarrier and allowed to dry about 2 minutes before bombardment.

20 Particle Gun Treatment

The sample plates are bombarded at level #4 in particle gun #HE34-1 or #HE34-2. All samples receive a single shot at 650 PSI, with a total of ten aliquots taken from each tube of prepared particles/DNA.

Subsequent Treatment

25 Following bombardment, the embryos are kept on 560Y medium for 2 days, then transferred to 560R selection medium containing 3 mg/liter Bialaphos, and subcultured every 2 weeks. After approximately 10 weeks of selection, selection-resistant callus clones are transferred to 288J medium to initiate plant regeneration. Following somatic embryo maturation (2-4 weeks), well-developed somatic embryos are transferred to 30 medium for germination and transferred to the lighted culture room. Approximately 7-10

days later, developing plantlets are transferred to 272V hormone-free medium in tubes for 7-10 days until plantlets are well established. Plants are then transferred to inserts in flats (equivalent to 2.5" pot) containing potting soil and grown for 1 week in a growth chamber, subsequently grown an additional 1-2 weeks in the greenhouse, then transferred

5   to classic 600 pots (1.6 gallon) and grown to maturity. Plants are monitored and scored for MSH2 biological activity.

EXAMPLE 8: *Agrobacterium*-mediated Transformation and
Regeneration of Transgenic Maize Plants

10

For *Agrobacterium*-mediated transformation of maize with an *MSH2* nucleotide sequence of the invention, preferably the method of Zhao is employed (PCT patent publication WO98/32326), the contents of which are hereby incorporated by reference. Briefly, immature embryos are isolated from maize and the embryos contacted with a

15   suspension of *Agrobacterium*, where the bacteria are capable of transferring the *MSH2* nucleotide sequence of interest to at least one cell of at least one of the immature embryos (step 1: the infection step). In this step the immature embryos are preferably immersed in an *Agrobacterium* suspension for the initiation of inoculation. The embryos are co-cultured for a time with the *Agrobacterium* (step 2: the co-cultivation step). Preferably

20   the immature embryos are cultured on solid medium following the infection step. Following this co-cultivation period an optional "resting" step is contemplated. In this resting step, the embryos are incubated in the presence of at least one antibiotic known to inhibit the growth of *Agrobacterium* without the addition of a selective agent for plant transformants (step 3: resting step). Preferably the immature embryos are cultured on

25   solid medium with antibiotic, but without a selecting agent, for elimination of *Agrobacterium* and for a resting phase for the infected cells. Next, inoculated embryos are cultured on medium containing a selective agent and growing transformed callus is recovered (step 4: the selection step). Preferably, the immature embryos are cultured on solid medium with a selective agent resulting in the selective growth of transformed cells.

The callus is then regenerated into plants (step 5: the regeneration step), and preferably calli grown on selective medium are cultured on solid medium to regenerate the plants.

EXAMPLE 9: Production of Transgenic Soybean Plants Using Embryo Transformation

Soybean embryos are bombarded with a plasmid containing *MSH2* nucleotide sequence of the invention operably linked to a SCP1 or UCP3 promoter as follows. To induce somatic embryos, cotyledons, 3-5 mm in length dissected from surface-sterilized, immature seeds of the soybean cultivar A2872, are cultured in the light or dark at 26°C on an appropriate agar medium for six to ten weeks. Somatic embryos producing secondary embryos are then excised and placed into a suitable liquid medium. After repeated selection for clusters of somatic embryos that multiplied as early, globular-staged embryos, the suspensions are maintained as described below.

Soybean embryogenic suspension cultures can maintained in 35 ml liquid media on a rotary shaker, 150 rpm, at 26°C with florescent lights on a 16:8 hour day/night schedule. Cultures are subcultured every two weeks by inoculating approximately 35 mg of tissue into 35 ml of liquid medium.

Soybean embryogenic suspension cultures may then be transformed by the method of particle gun bombardment (Klein *et al.* (1987) *Nature* (London) *327*:70-73, U.S. Patent No. 4,945,050). A Du Pont Biolistic PDS1000/HE instrument (helium retrofit) can be used for these transformations.

A selectable marker gene that can be used to facilitate soybean transformation is a transgene composed of the 35S promoter from Cauliflower Mosaic Virus (Odell *et al.* (1985) *Nature 313*:810-812), the hygromycin phosphotransferase gene from plasmid pJR225 (from *E. coli*; Gritz *et al.* (1983) *Gene 25*:179-188), and the 3' region of the nopaline synthase gene from the T-DNA of the Ti plasmid of *Agrobacterium tumefaciens*. The expression cassette comprising the *MSH2* nucleotide sequence of the invention operably linked to the SCP1 or UCP3 promoter can be isolated as a restriction fragment. This fragment can then be inserted into a unique restriction site of the vector carrying the marker gene.

To 50 μl of a 60 mg/ml 1 μm gold particle suspension is added (in order): 5 μl DNA (1 μg/μl), 20 μl spermidine (0.1 M), and 50 μl $CaCl_2$ (2.5 M). The particle preparation is then agitated for three minutes, spun in a microfuge for 10 seconds and the supernatant removed. The DNA-coated particles are then washed once in 400 μl 70%

5 ethanol and resuspended in 40 μl of anhydrous ethanol. The DNA/particle suspension can be sonicated three times for one second each. Five microliters of the DNA-coated gold particles are then loaded on each macro carrier disk.

Approximately 300-400 mg of a two-week-old suspension culture is placed in an empty 60x15 mm petri dish and the residual liquid removed from the tissue with a

10 pipette. For each transformation experiment, approximately 5-10 plates of tissue are normally bombarded. Membrane rupture pressure is set at 1100 psi, and the chamber is evacuated to a vacuum of 28 inches mercury. The tissue is placed approximately 3.5 inches away from the retaining screen and bombarded three times. Following bombardment, the tissue can be divided in half and placed back into liquid and cultured

15 as described above.

Five to seven days post bombardment, the liquid media may be exchanged with fresh media, and eleven to twelve days post-bombardment with fresh media containing 50 mg/ml hygromycin. This selective media can be refreshed weekly. Seven to eight weeks post-bombardment, green, transformed tissue may be observed growing from

20 untransformed, necrotic embryogenic clusters. Isolated green tissue is removed and inoculated into individual flasks to generate new, clonally propagated, transformed embryogenic suspension cultures. Each new line may be treated as an independent transformation event. These suspensions can then be subcultured and maintained as clusters of immature embryos or regenerated into whole plants by maturation and

25 germination of individual somatic embryos.

EXAMPLE 10: Production of Transgenic Sunflower Plants

Using Meristem Tissue Transformation

Sunflower meristem tissues are transformed with an expression cassette

5   containing an *MSH2* nucleotide sequence of the invention operably linked to a SCP1

promoter as follows (see also European Patent Number EP 0 486233, herein incorporated

by reference, and Malone-Schoneberg *et al.* (1994) *Plant Science 103*:199-207). Mature

sunflower seed (*Helianthus annuus* L.) are dehulled using a single wheat-head thresher.

Seeds are surface sterilized for 30 minutes in a 20% Clorox bleach solution with the

10   addition of two drops of Tween 20 per 50 ml of solution. The seeds are rinsed twice with

sterile distilled water.

Split embryonic axis explants are prepared by a modification of procedures

described by Schrammeijer *et al.* (Schrammeijer *et al.*(1990) *Plant Cell Rep. 9*: 55-60).

Seeds are imbibed in distilled water for 60 minutes following the surface sterilization

15   procedure. The cotyledons of each seed are then broken off, producing a clean fracture at

the plane of the embryonic axis. Following excision of the root tip, the explants are

bisected longitudinally between the primordial leaves. The two halves are placed, cut

surface up, on GBA medium consisting of Murashige and Skoog mineral elements

(Murashige *et al.* (1962) *Physiol. Plant., 15*: 473-497), Shepard's vitamin additions

20   (Shepard (1980) in *Emergent Techniques for the Genetic Improvement of Crops*

(University of Minnesota Press, St. Paul, Minnesota), 40 mg/l adenine sulfate, 30 g/l

sucrose, 0.5 mg/l 6-benzyl-aminopurine (BAP), 0.25 mg/l indole-3-acetic acid (IAA), 0.1

mg/l gibberellic acid (GA3), pH 5.6, and 8 g/l Phytagar.

The explants are subjected to microprojectile bombardment prior to

25   *Agrobacterium* treatment (Bidney *et al.* (1992) *Plant Mol. Biol. 18*: 301-313). Thirty to

forty explants are placed in a circle at the center of a 60 X 20 mm plate for this treatment.

Approximately 4.7 mg of 1.8 μm tungsten microprojectiles are resuspended in 25 ml of

sterile TE buffer (10 mM Tris HCl, 1 mM EDTA, pH 8.0) and 1.5 ml aliquots are used

per bombardment. Each plate is bombarded twice through a 150 mm nytex screen placed

30   2 cm above the samples in a PDS 1000® particle acceleration device.

Disarmed *Agrobacterium tumefaciens* strain EHA105 is used in all transformation experiments. A binary plasmid vector comprising the expression cassette that contains the *MSH2* nucleotide sequence of the invention operably linked to the SCP1 promoter is introduced into *Agrobacterium* strain EHA105 via freeze-thawing as described by

5 Holsters *et al.* (1978) *Mol. Gen. Genet.* *163*:181-187. This plasmid further comprises a kanamycin selectable marker gene (i.e, *nptII*). Bacteria for plant transformation experiments are grown overnight (28°C and 100 RPM continuous agitation) in liquid YEP medium (10 gm/l yeast extract, 10 gm/l Bactopeptone, and 5 gm/l NaCl, pH 7.0) with the appropriate antibiotics required for bacterial strain and binary plasmid

10 maintenance. The suspension is used when it reaches an OD_{600} of about 0.4 to 0.8. The *Agrobacterium* cells are pelleted and resuspended at a final OD_{600} of 0.5 in an inoculation medium comprised of 12.5 mM MES pH 5.7, 1 gm/l NH_4Cl, and 0.3 gm/l $MgSO_4$.

Freshly bombarded explants are placed in an *Agrobacterium* suspension, mixed,

15 and left undisturbed for 30 minutes. The explants are then transferred to GBA medium and co-cultivated, cut surface down, at 26°C and 18-hour days. After three days of co-cultivation, the explants are transferred to 374B (GBA medium lacking growth regulators and a reduced sucrose level of 1%) supplemented with 250 mg/l cefotaxime and 50 mg/l kanamycin sulfate. The explants are cultured for two to five weeks on selection and then

20 transferred to fresh 374B medium lacking kanamycin for one to two weeks of continued development. Explants with differentiating, antibiotic-resistant areas of growth that have not produced shoots suitable for excision are transferred to GBA medium containing 250 mg/l cefotaxime for a second 3-day phytohormone treatment. Leaf samples from green, kanamycin-resistant shoots are assayed for the presence of NPTII by ELISA and for the

25 presence of transgene expression by assaying for MSH2 activity. See, Su *et al.* ((1988*) J. Biol. Chem.* 263:6829-6835), Holmes *et al.* ((1990) *Proc. Natl. Acad. Sci.* USA 87:5837-5841), and Rice *et al.* ((2000) *Plant Physiol.* 123:427-438).

NPTII-positive shoots are grafted to Pioneer® hybrid 6440 *in vitro*-grown sunflower seedling rootstock. Surface sterilized seeds are germinated in 48-0 medium

30 (half-strength Murashige and Skoog salts, 0.5% sucrose, 0.3% gelrite, pH 5.6) and grown

under conditions described for explant culture. The upper portion of the seedling is removed, a 1 cm vertical slice is made in the hypocotyl, and the transformed shoot inserted into the cut. The entire area is wrapped with Parafilm to secure the shoot. Grafted plants can be transferred to soil following one week of *in vitro* culture. Grafts in

5 soil are maintained under high humidity conditions followed by a slow acclimatization to the greenhouse environment. Transformed sectors of T_0 plants (parental generation) maturing in the greenhouse are identified by NPTII ELISA and/or by MSH2 activity analysis of leaf extracts while transgenic seeds harvested from NPTII-positive T_0 plants are identified by MSH2 activity analysis of small portions of dry seed cotyledon.

10 An alternative sunflower transformation protocol allows the recovery of transgenic progeny without the use of chemical selection pressure. Seeds are dehulled and surface-sterilized for 20 minutes in a 20% Clorox bleach solution with the addition of two to three drops of Tween 20 per 100 ml of solution, then rinsed three times with distilled water. Sterilized seeds are imbibed in the dark at 26°C for 20 hours on filter

15 paper moistened with water. The cotyledons and root radical are removed, and the meristem explants are cultured on 374E (GBA medium consisting of MS salts, Shepard vitamins, 40 mg/l adenine sulfate, 3% sucrose, 0.5 mg/l 6-BAP, 0.25 mg/l IAA, 0.1 mg/l GA, and 0.8% Phytagar at pH 5.6) for 24 hours under the dark. The primary leaves are removed to expose the apical meristem, around 40 explants are placed with the apical

20 dome facing upward in a 2 cm circle in the center of 374M (GBA medium with 1.2% Phytagar), and then cultured on the medium for 24 hours in the dark.

Approximately 18.8 mg of 1.8 µm tungsten particles are resuspended in 150 µl absolute ethanol. After sonication, 8 µl of it is dropped on the center of the surface of macrocarrier. Each plate is bombarded twice with 650 psi rupture discs in the first shelf

25 at 26 mm of Hg helium gun vacuum.

The plasmid of interest is introduced into *Agrobacterium tumefaciens* strain EHA105 via freeze thawing as described previously. The pellet of overnight-grown bacteria at 28 °C in a liquid YEP medium (10 g/l yeast extract, 10 g/l Bactopeptone, and 5 g/l NaCl, pH 7.0) in the presence of 50 µg/l kanamycin is resuspended in an

30 inoculation medium (12.5 mM 2-(N-morpholino) ethanesulfonic acid, MES, 1 g/l NH_4Cl

and 0.3 g/l MgSO$_4$ at pH 5.7) to reach a final OD6$_{00}$ of 4.0. Particle-bombarded explants are transferred to GBA medium (374E), and a droplet of bacteria suspension is placed directly onto the top of the meristem. The explants are co-cultivated on the medium for 4 days, after which the explants are transferred to 374C medium (GBA with 1% sucrose

5   and no BAP, IAA, GA3 and supplemented with 250 μg/ml cefotaxime). The plantlets are cultured on the medium for about two weeks under 16-hour day and 26°C incubation conditions.

Explants (around 2 cm long) from two weeks of culture in 374C medium are screened for expression of the selectable marker gene and then those that are positive for

10   expression of the marker gene are then screened for MSH2 activity using assays known in the art. After positive (i.e., for *MSH2* expression) explants are identified, and every positive explant is subdivided into nodal explants. One nodal explant contains at least one potential node. The nodal segments are cultured on GBA medium for three to four days to promote the formation of auxiliary buds from each node. Then they are

15   transferred to 374C medium and allowed to develop for an additional four weeks. Developing buds are separated and cultured for an additional four weeks on 374C medium. Pooled leaf samples from each newly recovered shoot are screened again by the appropriate protein activity assay. At this time, the positive shoots recovered from a single node will generally have been enriched in the transgenic sector detected in the

20   initial assay prior to nodal culture.

Recovered shoots positive for *MSH2* expression are grafted to Pioneer hybrid 6440 *in vitro*-grown sunflower seedling rootstock. The rootstocks are prepared in the following manner. Seeds are dehulled and surface-sterilized for 20 minutes in a 20% Clorox bleach solution with the addition of two to three drops of Tween 20 per 100 ml of

25   solution, and are rinsed three times with distilled water. The sterilized seeds are germinated on the filter moistened with water for three days, then they are transferred into 48 medium (half-strength MS salt, 0.5% sucrose, 0.3% gelrite pH 5.0) and grown at 26 °C under the dark for three days, then incubated at 16-hour-day culture conditions. The upper portion of selected seedling is removed, a vertical slice is made in each

30   hypocotyl, and a transformed shoot is inserted into a V-cut. The cut area is wrapped with

parafilm. After one week of culture on the medium, grafted plants are transferred to soil. In the first two weeks, they are maintained under high humidity conditions to acclimatize to a greenhouse environment.

5   All publications and patent applications mentioned in the specification are indicative of the level of those skilled in the art to which this invention pertains. All publications and patent applications are herein incorporated by reference to the same extent as if each individual publication or patent application was specifically and individually indicated to be incorporated by reference.

10   Although the foregoing invention has been described in some detail by way of illustration and example for purposes of clarity of understanding, it will be obvious that certain changes and modifications may be practiced within the scope of the appended embodiments.